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A *GH3*-like gene, *CcGH3*, isolated from *Capsicum chinense* L. fruit is regulated by auxin and ethylene★

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Abstract

Auxin, which has been implicated in multiple biochemical and physiological processes, elicits three classes of genes (*Aux/IAAs*, *SAUR*s and *GH3*s) that have been characterized by their early or primary responses to the hormone. A new *GH3*-like gene was identified from a suppressive subtraction hybridization (SSH) library of pungent pepper (*Capsicum chinense* L.) cDNAs. This gene, *CcGH3*, possessed several auxin- and ethylene-inducible elements in the putative promoter region. Upon further investigation, *CcGH3* was shown to be auxin-inducible in shoots, flower buds, sepals, petals and most notably ripening and mature pericarp and placenta. Paradoxically, this gene was expressed in fruit when auxin levels were decreasing, consistent with ethylene-inducibility. Further experiments demonstrated that *CcGH3* was induced by endogenous ethylene, and that transcript accumulation was inhibited by 1-methylcyclopropene, an inhibitor of ethylene perception. When over-expressed in tomato, *CcGH3* hastened ripening of ethylene-treated fruit. These results implicate *CcGH3* as a factor in auxin and ethylene regulation of fruit ripening and suggest that it may be a point of intersection in the signaling by these two hormones.

Introduction

Auxin is an essential plant hormone implicated in many processes including cell elongation, division and expansion, as well as embryogenesis, directional growth response (tropism), branching and meristem formation. Various auxin-regulated genes and cDNAs have been identified in plants using differential hybridization with probes from untreated and auxin-treated hypocotyls, epicotyls, or seedlings (Hagen *et al.*, 1984; Dargeviciute *et al.*, 1998). Among auxin-responsive genes, those that are specifically induced by auxin treatment within minutes of exposure are categorized as early, or primary auxin-response genes and fall into three major classes: *Aux/IAAs*, *SAURs* and *GH3*s (Hagen and Guilfoyle, 2002). In the *Arabidopsis* genome, at least 20 *GH3*-like genes

★ The nucleotide sequence reported will appear in the GenBank Nucleotide Sequence Database under the accession number AY525089.

have been identified based on annotation of genomic sequence, designated as *AtGH3-1* to *AtGH3-20* (Hagen and Guilfoyle, 2002). Several of these *AtGH3* genes have been cloned and shown to be auxin-inducible, except for *AtGH3-17*, which is constitutively expressed and does not respond to auxin (Hagen and Guilfoyle, 2002).

Two members of the *Arabidopsis GH3* gene family have been linked to phenotypes via mutant analysis. Results from these studies suggest that some *GH3* proteins are important in photomorphogenesis, strengthening the potential link between phytochrome signaling and auxin responses (Hsieh *et al*., 2000; Nakazawa *et al*., 2001). Beyond this observation, it remains unclear what roles, if any, the members of the *GH3* gene family play in plant growth or development. *Arabidopsis* plants that over-express a soybean *GH3* gene, *GmGH3*, display an exaggerated dwarf phenotype and a de-etiolated phenotype when germinated and grown in the dark (Hagen and Guilfoyle, 2002). Over-expression of another *GH3* gene from *Arabidopsis*, *FIN219/GH3-11*, resulted in shortened hypocotyls in plants grown under continuous far-red light, but dwarfing of adult plants was not reported (Hsieh *et al*., 2000). Clearly, ectopic expression of *GH3* genes can result in a variety of phenotypes suggesting non-redundant roles for gene family members in auxin and/or light signaling.

Endogenous expression of *GH3* genes has been studied in several systems. In both soybean and tobacco, the steady-state levels of *GmGH3* and *NtGH3* mRNA transcripts are undetectable in etiolated seedlings prior to auxin treatment, but these transcripts are rapidly induced within 10 min by exogenous auxin (Hagen *et al*., 1984; Roux and Perrot-Rechenmann, 1997). Induction of expression of the *GmGH3* gene and its promoter-GUS reporter gene occurs in most organs and tissues in a dose-dependent manner, indicating that auxin is a limiting factor for activation of the *GH3* gene (Li *et al*., 1999). Promoter analyses of several auxin-responsive genes (*GmGH3*, soybean *SAUR15A* and pea *PS-IAA4/5*) have identified a common auxin-responsive element (AuxRE), a six-base pair sequence, TGTCTC (Ulmasov *et al*., 1997a, c) or derivatives such as TGTCAC, found in the *PS-IAA4/5* promoter (Ballas *et al*., 1993, 1995). These elements have been shown to function both as members of composite elements and as a simple auxin-response element. Novel transcription factors, auxin-response factors (ARFs), have also been identified and shown to bind specifically to TGTCTC AuxREs (Ulmasov *et al*., 1997a, b, 1999). ARFs activate or repress transcription by binding to AuxREs directly and require a conserved dimerization domain found in both ARF and Aux/IAA proteins.

To date, *GH3* genes have been isolated only from mutant plants displaying seedling defects and auxin-treated etiolated seedlings. This study was undertaken to examine the role this gene family may play in reproductive organs, particularly in fruit development and ripening where auxin content is known to be a critical factor. Ovary growth and expansion subsequent to pollination depends upon a supply of auxin, which is typically derived from developing ovules and seeds (Gillaspy *et al*., 1993; Oneill, 1997). High auxin levels applied later in development, however, can delay fruit ripening in both climacteric and non-climacteric species (Given *et al*., 1988; Deruere *et al*., 1994; Aharoni *et al*., 2002; Balbi and Lomax, 2003). The application of auxin to strawberry fruit, a model for non-climacteric ripening, suppresses many ripening-associated genes (Manning, 1994, 1998; Harpster *et al*., 1998; Aharoni *et al*., 2002). Garden pepper (*Capsicum*) has typically been classified as non-climacteric, although varying patterns of ethylene and carbon dioxide production have been reported (Villavicencio *et al*., 1999; Watkins and Miller, 2004). In one study of the effects of auxin on pepper fruit ripening, exogenous indole-3-acetic acid (IAA) applied during fruit development delayed the synthesis of fibrillin and the accumulation of associated chromoplast carotenoids, the latter considered the hallmark of the onset of fruit ripening in pepper (Deruere *et al*., 1994).

Here we report the isolation and intial characterization of a *GH3*-like gene from the fruit of the pungent pepper 'Habenero' (*Capsicum chinense* cv. 'Habenero'). This gene, *CcGH3*, is shown to be auxin- and ethylene-inducible and controls a ripening phenotype when over-expressed in tomato fruit. Our results establish that this gene shows both the responses to auxin expected for this gene family and regulation consistent with a role in fruit ripening. Further analysis may define a basis for interaction between ethylene- and auxin-regulated processes in fruit development.

Materials and methods

Plant material

Pepper

For isolation of the gene and analysis of its expression in pepper, leaves, shoots, flower buds, open flowers and fruits of *C. chinense* cv. 'Habanero' were harvested from plants grown in temperature-controlled greenhouses (16 h light/8 h dark) held under supplemental light. Parthenocarpic fruits, which are much smaller and ripen earlier than normal, or self-pollinated, fruits were harvested.

Tomato

The *Solanum lycopersicon* cultivar 'Micro-Tom' was purchased from Ball Horticultural Company (West Chicago, IL, USA) and used for *Agrobacterium*-mediated transformation (See below). Plants were grown in a green house (16 h light/8 h dark).

Arabidopsis

Arabidopsis thaliana ecotypes 'Columbia (Col-0)' and 'Nisson' were used for this study. The ecotype Col-0 was used for *GH3* over-expression and Nisson was utilized to examine promoter activity. For measurement of hypocotyl lengths, *GH3*-transformed transgenic seeds and untransformed Col-0 seeds were surface-sterilized and plated on GM (1/2 strength MS) medium. Plated seeds were cold-treated at 4 °C for 72 h, and then transferred to darkness and incubated at 23 °C for 5 days. After incubation, hypocotyl lengths of all germinated seedlings were measured.

NAA treatments of seedlings, flower buds and flowers

Seeds were sterilized in 0.6% sodium hypochlorite (10% (v/v) Clorox) for 20 min, then rinsed with sterile water three times. The seeds were then placed on 1/2 X MS (Murashige and Skoog, 1962) media (0.3% agar) in magenta boxes and grown at 25 °C under 16 h light/8 h dark cycle for 2 weeks. Whole seedlings were washed briefly in distilled water, and transferred to 500 ml beakers containing 300 ml of 1/2 MS salt with or without 5 μM NAA for 3 h. Seedlings were then dissected into roots, hypocotyls and cotyledons, flash frozen in liquid nitrogen and stored in –80 °C. Flower buds and open flowers were collected from 'Habanero' plants grown in the greenhouse and soaked in 300 ml of MS salt with or without 5 μM NAA for 3 h, flash frozen in liquid nitrogen and stored in –80 °C.

Dissection of pepper flowers and ripe fruit

To investigate the expression profile of *CcGH3*, fully open flowers were collected and dissected into sepals, petals, stamens (anthers and filaments) and pistils (stigma, styles and ovaries). Ripe fruits were also harvested and dissected into pericarp, placenta and seeds. The dissected organs and tissues were flash frozen in liquid nitrogen and stored at –80 °C for RNA isolation.

In vitro *induction of ripening and treatment with ethylene and 1-MCP of mature green and ripe fruits*

Pericarp pieces (ca. 1 cm^2) were excised from mature green parthenocarpic fruits previously surface-sterilized as described above. Pericarp tissues were placed epidermis-side-down onto a plate containing 1/2 X MS media (0.3% agar) and cultured at 25 °C under 16 h light/8 h dark cycle to induce ripening and were collected for RNA isolation at 24, 48, 72 h and 10 days. Ethylene production of pepper fruit was measured using two sets of developmental stages. In the first set, fruit were harvested at the immature (average weight = 0.70 g), mature green (average weight = 4.0 g), and orange (average weight = 5.7 g) stages; in the second set, fruit from early (average weight = 5.3 g), mid (average weight = 5.0 g) and late (average weight = 5.0 g) turning stages were tested. Early, mid and late turning stages corresponded to 0.25, 0.5 and 0.75 of the surface area of the fruit being orange. These stages were designated $T_{0.25}$, $T_{0.5}$ and $T_{0.75}$, respectively.

Five fruits of each developmental stage were sealed individually in 130 ml glass containers fitted with a sampling septum for 5 h. An empty container was similarly sealed to ensure that background ethylene was negligible. One millilitre samples of the headspace of each container were taken, and the ethylene concentration was measured using a gas chromatograph fitted with a flame ionization detector (Hewlett Packard 5890, series II, Wilmington, DE). Differences among

means were analyzed separately for each series by one-way ANOVA. For ethylene treatment, mature green pericarp tissues obtained as above and maintained on culture plates were put in air-tight plastic containers fitted with septa. Ethylene (2 ml) from a stock gas cylinder was injected to provide a concentration of 20 μl l^{-1}. Ethylene concentrations were verified by gas chromatography and remained constant over the experimental period. The containers were then kept at 25 °C in the dark. The pericarp tissues were collected for RNA isolation and subsequent Northern analysis after 0, 2, 4, 6, 10, 24, 48, and 72 h of treatment.

For treatment with the ethylene inhibitor, 1-methylcyclopropene (1-MCP), pericarp tissue from both mature green fruits and ripe fruits on culture plates prepared as described above were put in air-tight containers. A stock 1-MCP solution prepared from SmartFresh® powder (AgroFresh Inc., Rohm and Haas, Spring House, PA) dissolved in water was injected to provide a final concentration of 20 μl l^{-1}. Concentrations were verified by gas chromatography and declined over time to reach about 10 μl l^{-1} by 72 h. Pericarp tissues were collected for RNA isolation and subsequent Northern analysis after 0, 2, 4, 6, 10, 24, 48, and 72 h of treatment. For both ethylene and 1-MCP treatments, pericarp tissue from the same fruit used for the treatments were also placed on the culture plates treated with air for the same periods of time to serve as controls.

Transient expression of auxin-inducible DR5: GUS gene in young and ripe fruits

Immature and mature pepper fruits were surface-sterilized with 0.6% sodium hypochlorite for 20 min, then rinsed with sterilized distilled water three times. Pericarp tissue was excised and immediately transferred onto plates of MS medium without indole-acetic acid (IAA) treatment, or were soaked in MS liquid medium containing IAA at 500 μg l^{-1} for 3 h before transfer to plates containing solid MS medium and 500 mg IAA 1^{-1}. The plates were incubated under light at 25 °C for 20 h. A *DR5:GUS* reporter construct (Ulmasov *et al.*, 1997c) was delivered into the pericarp cells using the Bio-RAD biolistic particle delivery system. Each sample had three duplicate plates respectively, and each plate was bombarded twice. After bombardment, plates were incubated under light at 25 °C for 24 h. The pepper pieces were stained as described previously (Jefferson, 1987).

RNA Extraction and Northern analysis

Total RNA was extracted from all treated and untreated tissues described above using TRIZOL® reagent and the manufacturer's recommended protocol (Invitrogen, Carlsbad, CA). Poly A^+ RNA was isolated from total RNA using oligo $d(T)_{25}$ Dynalbeads (Dynal). Total RNA samples were quantified spectrophotometrically, 10 μg fractionated on 1% formaldehyde-agarose gel in MOPS (morpholinopropanesulfonic acid) buffer and blotted to Hybond-N^+ (Amersham, Piscataway, NJ) membrane with 10 × SSC as the transfer solution. RNA was cross-linked to the filters by UV radiation. Northern blots were prehybridized in Church buffer (7% SDS, 1% bovine serum albumin, 1 mM EDTA, Na_2HPO_4 0.25 M, pH 7.2) at 65 °C for 3 h and hybridized in Church buffer with the full-length *CcGH3* cDNA for 16 h. The filters were then washed at 65 °C with 2 × SSC, 0.1% SDS for 30 min, then 0.5 × SSC, 0.1% SDS for 30 min. Filters were then exposed to X-ray film or PhosphorImager screen for 1–3 days. Equal loading of RNA samples were confirmed by ethidium bromide staining before blotting or by hybridization with 18S RNA gene after initial probes were stripped from the blots. The probes were stripped at 65 °C for 30 min with boiling 0.1% SDS.

Rapid amplification of cDNA 5′ and 3′ ends (RACE)

Due to the intrinsic features of the SSH method, the original cDNA clone identified from the library was a partial sequence. To obtain the full-length cDNA, one μg of poly A^+ RNA isolated from ripe fruits was reverse-transcribed with RNase H^- MMLV reverse transcriptase (New England Biolabs, Beverly, MA) using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). Both 5′- and 3′-rapid amplification of cDNA ends (RACE) were performed according to the manufacturer's manual. The CLONTECH SMART technology provides a mechanism for generating full-length cDNAs in reverse transcription reactions (Zhu *et al.*, 2001). The resultant first-strand cDNAs were amplified

with primers 12F4-3RACE (ACCCGCGTGGTCGAGTACACTAGTTATGC) and 12F4-5RACE (CGACGTCGTTCAGGTGTCCATTGAGGC), respectively, using Advantage 2 polymerase mixture (Clontech, Palo Alto, CA). The PCR bands were purified with Qiagen Gel Purification Kit and cloned into pGEM-T Easy vector. At least three independent positive clones for each fragment were sequenced and assembled to avoid errors introduced in PCR reactions.

The GH3 amino acid sequences were aligned using DiAlign on the Genomatix web server (Morgenstern *et al.*, 1996, 1998) (http://www.genomatix.de) using diagonal similarity and threshold = 0. The resulting alignment was analyzed with the PHYLIP phylogeny package (Felsenstein, 1989) (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html). PROTDIST was used to compute a distance matrix based on the input alignment based on the Dayhoff PAM matrix. PROTPARS was used to produce an unrooted phylogeny based on parsimony criteria. In each case the input alignment was bootstrapped before analysis with 100 replicates. The neighbor-joining algorithm BioNJ (Gascuel, 1997) was used to construct trees. CONSENSE was used to construct majority rule consensus trees for each of these datasets. The two resulting trees were combined again with CONSENSE to produce a strict consensus tree that showed agreement between the two phylogenetic methods. Trees were drawn using TreeView (Page, 1996).

RT-PCR

To further verify the *CcGH3* gene expression profiles detected in fruit development and different tissues via Northern analysis, reverse transcriptase-PCR (RT-PCR) method was employed to amplify the transcripts of *CcGH3* from leaves, flower buds, open flowers, shoots and fruits at five day intervals. One microgram of total RNA was treated with one unit of amplification grade DNase I (Invitrogen, Carlsbad, CA) in 10 μl reaction at room temperature for 15 min, then heated at 65 °C for 10 min to inactivate the DNase I. The DNase I-treated total RNA was then reverse-transcribed with 1 μg of 10-mer random primers (New England Biolabs, Beverly, MA) using 200 units of RNase H^- MMLV reverse-transcriptase (New England Biolabs, Beverly, MA) in a 20 μl reaction at 42 °C for 2 h. The resultant first-strand cDNAs were diluted into 50 μl with 30 μl of water. A 392 bp fragment was amplified with the two *CcGH3* specific primers 12F4-3RACE and 12F4-5RACE as described above using the following PCR cycles: 94 °C 30 s, 62 °C 30 s, 72 °C 1 min for 22 cycles. The 18S rRNA gene-specific primers and Competimers® of the Quantum RNA 18S Internal Standards Kit (Ambion, Austin, TX) were added to the same RT-PCR reactions as internal standards for RNA quantity. The specifically modified competimers could anneal to the 18S rRNA templates and compete for the binding sites with the regular 18S rRNA primers but could not be extended, resulting in the production of an attenuated 315 bp internal fragment.

Isolation of the upstream promoter region

Four GenomeWalker libraries were constructed for isolating the upstream promoter region using the Genomewalker kit (Clontech, Palo Alto, CA) according to the manufacturer's manual. 'Habanero' pepper genomic DNA was digested with the restriction enzymes *Dra*I, *Eco*RV, *Hpa*I and *Sca*I, all six base cutters that produced blunt ends after digestion. Following digestion, each pool of DNA fragments was ligated to the GenomeWalker Adaptor. The upstream genomic region was then amplified from each library using two nested adaptor primers and two nested gene-specific primers. The primary PCR amplification used the outer adaptor primer (AP1) provided in the kit and the outer gene-specific primer 12F4NEST1 (TTCA CATGCAGGAGGACCCAATGGAGATG).One microlitre of the primary PCR mixture was then diluted to 50 μl, and 1 μl of the diluted primary PCR mixture was used as a template for the secondary or "nested" PCR amplification using the nested adaptor primer (AP2) and the nested gene-specific primer 12F4NEST2 (GGACCCAATGGA GATGAAAGTACTGAATCAACAG) or 12F4N EST3 (CTTAAGAGAGTTGGGAGAGTTGTG TATGG). All three gene-specific primers were designed based on the 150 bp 5′ end of the full-length cDNA including the 99-bp 5′ UTR. Two nested primers, 12F4NEST2 and 12F4NEST3, which are approximately 100-bp apart, were used for the secondary PCR amplification, respectively. Each primer produced a single major PCR product of 1.4 and 1.3 kb, respectively, which were

amplified from the *Eco*RV library using the 12F4NEST2 and 12F4NEST3. These two bands were purified, cloned into the pGEM-T easy vector and sequenced as previously above.

Generation of transgenic plants

Tomato

The full-length ORFs of *GH3* gene was PCR amplified from 5′ RACE-ready cDNA of Habanero using primers AuxBamHI (5′CGCGGATCCA-TGGCTGTTGATTCAGTAGTACT 3′) and AuxSacI (5′ACGAGCTCAACGACGTCGTTC-AGGTG 3′), cloned into pGEM-T Easy vector (Promega, Madison, WI) and verified by sequencing. The full-length ORF was cut from the pGEM-T Easy vector with BamHI and SacI, and replaced with the GUS gene in the binary vector pBI121. The resulting plasmid construct was designated pKL8. In addition to the constructs, the GUS gene of pBI121 was removed and used as negative control for transformation. The construct pKL8 and the empty vector pBI121 were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation and the transformed *Agrobactria* were used for tomato transformation. 'Micro-Tom' cotyledons were transformed and regenerated into whole plants as described earlier (Frary and Earle, 1996). Seven independent transgenic plants were obtained from pKL8 construct and 14 transgenic plants were obtained from vector alone plasmid. *GH3* gene expression was tested by Northern blot analysis as stated above. In order to investigate ethylene responses of transgenic tomato fruits, mature green fruits (30 dpa) of approximately same size were harvested. Fruit were placed in a sealed chamber equipped with a gasket and gassed with 20 ppm l l^{-1}ethylene for 8 h.

Arabidopsis

To study promoter activity of pepper *GH3*, primers D1F (5′ATCATCGTATATGGTTAATT 3′) and D1R (5′GCGGAGGATGGAGTAAGAG G3′) were used to amplify a 2 kb region upstream of the start of transciption of the *GH3* gene. The PCR products were digested by *Bam*HI and *Sma*I, and subcloned into the pBI121 vector. The promoter-GUS construct was designated pD1 and was made as described above.

The transformation protocol utilized for *in planta* transformation of *Arabidopsis* was as described by Clough and Bent (1998). The constructs pKL8 and pD1 were introduced into *A. tumefaciens* strain C58C1 by electroporation and transferred to *Arabidopsis thaliana* (Columbia) by vaccum infiltration. The selection of putative transformants was done on 80 mg l^{-1} kanamycin-containing agar medium. For GUS activity assay, seedlings or open flowers of transgenic plants bearing promoter-GUS constructs were stained according to previously described protocol (Jefferson, 1987).

Results

Identification of an auxin-responsive GH3*-like mRNA by suppressive subtraction hybridization and sequence analysis of* CcGH3

To identify fruit-specific and/or ripening-induced genes in pepper, a suppressive subtraction hybridization (SSH) cDNA library was made using ripe fruit mRNA as the tester and leaf mRNA as the driver (Diatchenko *et al.*, 1996). Five hundred randomly picked cDNA clones were sequenced and annotated. At least 125 clones matched fruit related sequences identified in previous studies or were shown to be fruit specific by Northern blot analysis (Liu and Jahn, unpublished data). Two clones, in particular, had identical sequences and shared a high degree of similarity with auxin-responsive *GH3*-like genes. RACE was performed to obtain the full-length cDNA sequence. PCR products of 1.9 and 0.7 kb were obtained for both 5′ and 3′ ends, respectively, cloned, sequenced, and aligned with the original cDNA sequences into a single 2180 bp contig with an overlap of 392 bp among the three sequences with perfect matches. The contig contained the full-length cDNA including the transcription start point, a potential open reading frame of 1788 bp, a 5′-untranslated region (5′-UTR) of 99 bp and a 3′-UTR of 293 bp including the poly (A) tail.

The predicted open reading frame encodes 595 amino acid residues with a molecular mass of 67.43 kDa. The calculated isoelectric point is 6.37. Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982) indicates that the putative protein is mainly composed of hydrophilic residues and is likely to be soluble. There are no significant known motifs or domains in the deduced amino acid

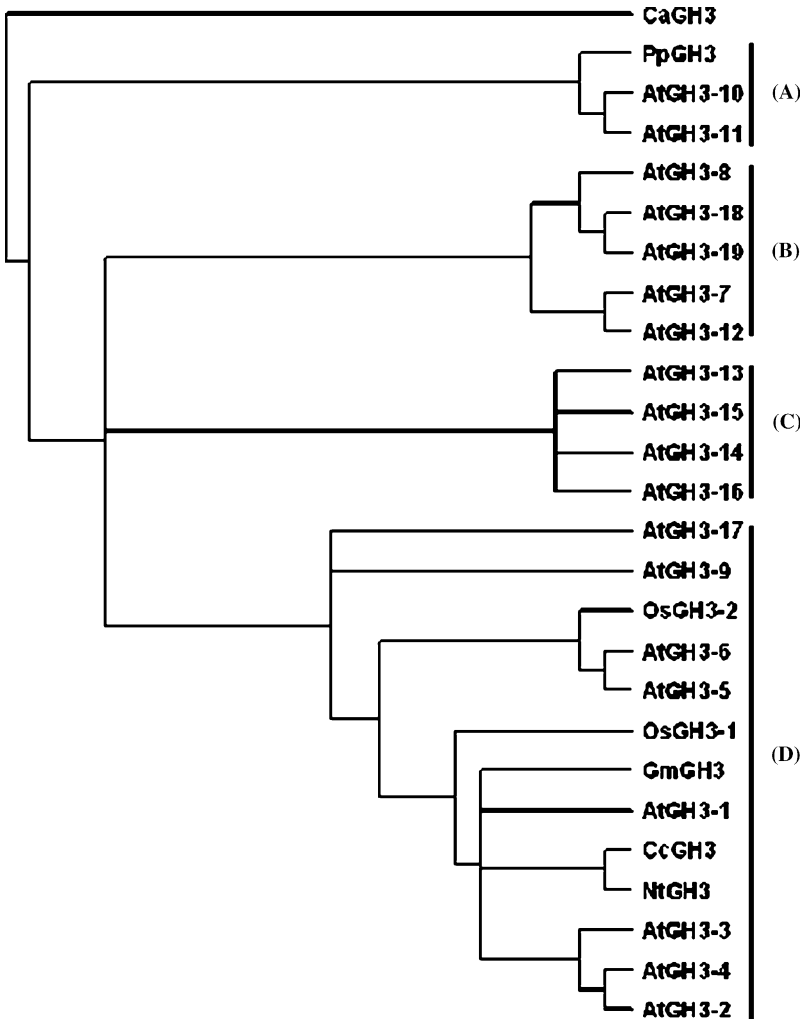

Figure 1. Strict consensus dendrogram showing *CcGH3* (AY525089) with all known GH3-like sequences from plants. The following protein sequences from Genbank were aligned with DIALIGN and analyzed by both the PROTDIST and PROTPARS methods in the PHYLIP package. Genbank accession numbers follow the species of origin and gene designation: *Arabidopsis thaliana*, *AtGH3–1* to *AtGH3–20*: AAC61292, CAB38206, AAB87114, AAF79776, CAA19720, BAA97524, AAC00604, BAB08663, AAC63630, AAD14468, AAD23040, CAB86639, CAB86642, CAB87143, CAB87144, CAB87145, AAF98442, AAG60120, AAG60122 and AAG60118; *Oryza sativa*, *OsGH3-1*, BAB63594; *OsGH3-2*, BAB92590; *Physcomitrella patens, PpGH3*, BAB71764; *Nicotiana tabacum*, *NtGH3*, AAD32141; *Glycine max, GmGH3*, S17433. *Clostridium acetobutylicum*, *CaGH3*, NP_350001, served as the outgroup.

sequence of pepper *GH3* gene (Hagen and Guilfoyle, 2002). The PSORT program predicted that the pepper GH3 protein would be located in the cytoplasm (Nakai and Horton, 1999).

Comparison between CcGH3 *and other* GH3-*like genes*

The deduced full-length amino acid sequence of pepper *GH3* (AY525089) was used to search translated sequences in GenBank (Altschul *et al*., 1997). Database searches have identified a number of *GH3*-like genes from tobacco (AAD32141), soybean (S17433), *Arabidopsis* (*AtGH3-1* to *AtGH3-20*: AAC61292, CAB38206, AAB87114, AAF79776, CAA19720, BAA97524, AAC00604, BAB08663, AAC63630, AAD14468, AAD23040, CAB86639, CAB86642, CAB87143, CAB87144, CAB87145, AAF98442, AAG60120, AAG60122 and AAG60118), rice (BAB63594 and BAB63594)

and moss (*Physcomitrella patens*) (BAB71764) (Imaizumi *et al*., 2002) and from the genome sequences of the bacterium *Clostridium acetobutylicum* (NP350001) (Nolling *et al*., 2001). We have designated these *GH3*-related genes using the species from which they were isolated (Roux and Perrot-Rechenmann, 1997; Hagen and Guilfoyle, 2002), and therefore, the pepper cDNA sequence was named *CcGH3* (*C. chinense*) and for clarity the soybean (*Glycine max*) *GH3* sequence was renamed *GmGH3*. The two putative rice proteins predicted from genome sequences were also re-designated *OsGH3-1* and *OsGH3-2* and the *GH3*-like sequence found in *C. acetobutylicum* (Nolling *et al*., 2001) was named *CaGH3*.

The overall identities between *CcGH3* and other *GH3*-like proteins range from 37% to 95%. The most similar sequence is the tobacco auxin-responsive gene *NtGH3* (Roux and Perrot-Rechenmann, 1997) whose predicted protein shares 95% identity and 96% similarity with the putative pepper GH3 protein. The identity and similarity between pepper *GH3* and the well-characterized soybean *GmGH3* (Hagen *et al*., 1984) are 73% and 85%, respectively. All the *GH3* genes encode proteins with predicted molecular masses of 65–70 kDa and have no characterized domains except the *FIN219*/*GH3-11* protein, which has putative coiled-coil domains in both amino- and carboxyl-terminal regions (Hsieh *et al*., 2000).

A dendrogram was constructed for *CcGH3* and the other 25 full-length plant, moss and bacterial GH3 predicted proteins (Figure 1). These proteins grouped into four major clades. Clade A included predicted proteins for two *Arabidopsis GH3*-like genes, *AtGH3-10* and *FIN219*/*AtGH3-11*, and the *P. patens* GH3-like protein 1 *(PpGH3L1)*. No members of Clades B and C have been characterized. Clade D included the auxin-inducible *GmGH3*, *NtGH3* and *CcGH3* genes and *DFLI*/*AtGH3-6*, an auxin-responsive *GH3* homologue which negatively regulates shoot cell elongation and lateral root formation and also positively regulates the light response of hypocotyl length in *Arabidopsis* (Nakazawa *et al*., 2001).

CcGH3 *is induced by auxin*

The strong homology between *CcGH3* and a number of early auxin-inducible *GH3* genes prompted us to examine whether the transcript levels of *CcGH3* mRNA were also auxin-inducible. Transcript levels of *CcGH3* in roots, hypocotyls and cotyledons of pepper seedlings, flower buds and open flowers treated with and without α-naphthalene acetic acid (NAA) were compared via RNA blot hybridization. Steady-state levels of *CcGH3* mRNA transcripts were low or undetectable in roots, hypocotyls and cotyledons but increased after NAA treatment (Figure 2A). The strongest induction occurred in roots, suggesting that these tissues have the greatest sensitivity to auxin. The lowest level of induction occurred in cotyledons. In flower buds and open flowers, accumulation of *CcGH3* mRNA transcripts was substantial in the absence of exogenous auxin, but was increased two to threefold by NAA treatment (Figure 2B). These experiments indicate that *CcGH3* is auxin-inducible, as similarly demonstrated for other *GH3*s in soybean (Hagen *et al*., 1984), tobacco (Roux and Perrot-Rechenmann, 1997) and *Arabidopsis* (Hsieh *et al*., 2000; Nakazawa *et al*., 2001). Further experiments would be required to determine if the effect of auxin in this system is direct or indirect (e.g., as a result of auxin-induced ethylene production).

To determine the kinetics of induction, seedlings were treated with 5 μM NAA for periods from 10 min to 24 h. Steady-state levels of *CcGH3* were very low in untreated seedlings, but were clearly induced within 30 min of NAA treatment. The transcript level increased considerably after 6 h of NAA treatment and remained high when treatments continued up to 24 h (Figure 2C). These data contrast with those for soybean and tobacco *GH3* mRNAs, where accumulation of *GmGH3* and *NtGH3* transcripts rapidly but transiently increased in response to auxin stimulation (Hagen *et al*., 1984; Roux and Perrot-Rechenmann, 1997; Li *et al*., 1999). *CcGH3* transcript accumulation was roughly proportional to NAA concentrations from 10^{-9} to 10^{-5} M (Roux and Perrot-Rechenmann, 1997; Li *et al*., 1999) (Figure 2D).

Expression profile of CcGH3 *in Capsicum plants*

To investigate further the expression of *CcGH3* in reproductive organs, *CcGH3* mRNA levels were compared in sepals, petals, stamens (including anthers and filaments) and pistils (including stigma, styles and ovaries). *CcGH3* transcripts

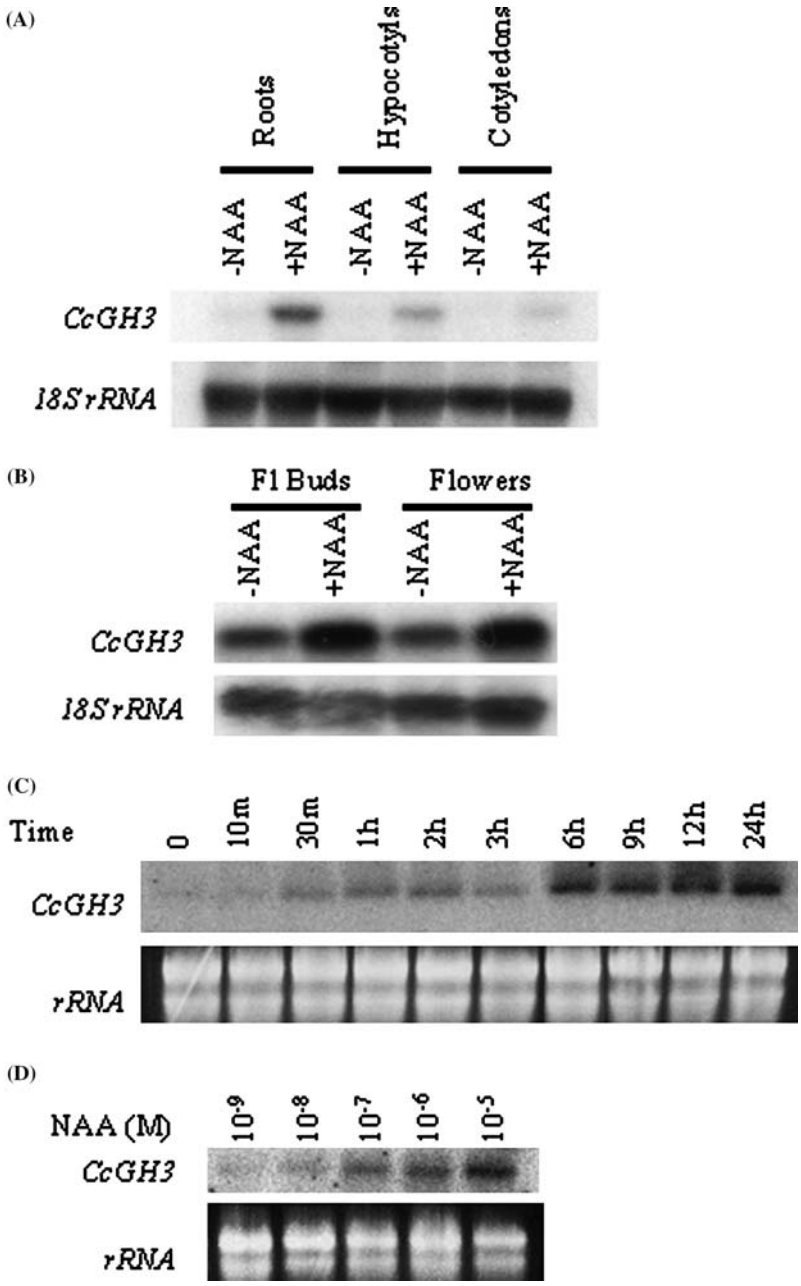

Figure 2. Induction of *CcGH3* mRNA transcripts by treatment with NAA. Tissues from *C. chinense* Habanero plants were treated with NAA and assayed for expression of *CcGH3* by RNA blot hybridization. After hybridizations, the blots were hybridized with 18S ribosomal RNA probe as a loading standard. (A) *CcGH3* expression in 2-week old seedlings treated with 5 mM NAA for 3 h, then dissected into roots, hypocotyls and cotyledons. (B) *CcGH3* expression in flower buds and flower tissues treated with 5 μM NAA for 3 h. (C) Time course of *CcGH3* transcript induction in 2-week old seedlings treated with 5 μM NAA. (D) Two-week old seedlings were treated for 3 h with NAA at noted concentrations.

were detected only in sepals and petals and not in stamens and pistils (Figure 3A). Steady-state levels of *CcGH3* mRNA transcripts were also assayed in mature leaves, shoots, flower buds, and open flowers as described above, and in fruit at 5-day intervals from 5 days post anthesis (dpa) to 60 dpa (Figure 3B). Transcripts were barely detectable in developing fruits from 2 to 35 dpa

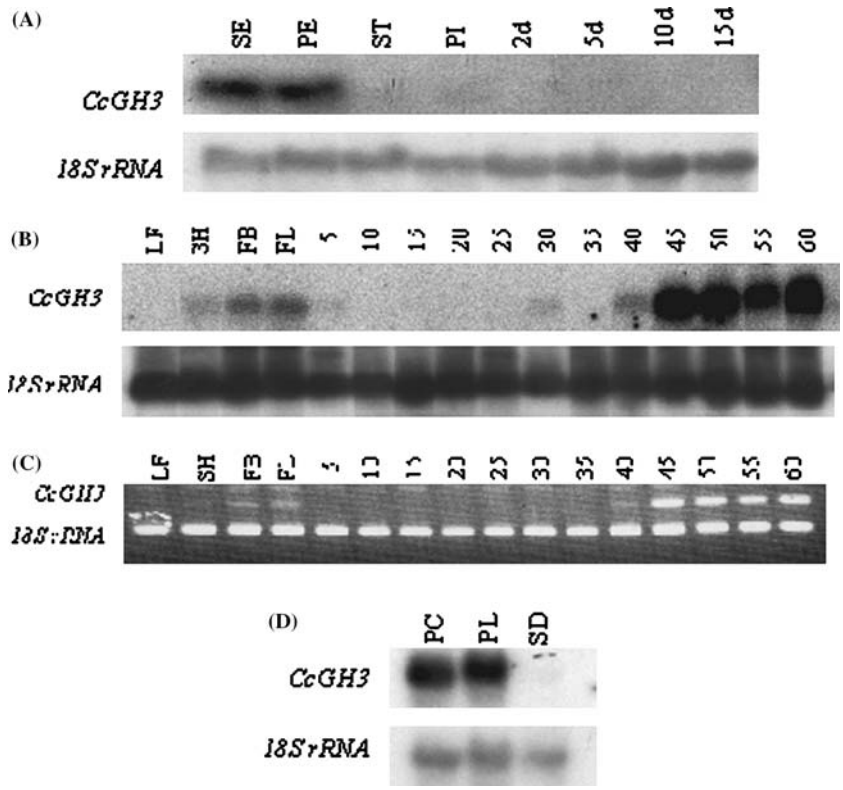

Figure 3. Localization of *CcGH3* expression in *C. chinense* Habanero flower and fruit tissue and its accumulation through fruit development. *CcGH3* mRNA transcripts were detected by RNA blot analysis or RT-PCR with 18S ribosomal RNA used as a loading control. (A) Accumulation of *CcGH3* transcript was assayed by RNA blot hybridization in sepals (SE), flower petals (PE), stamens including anthers and filaments (ST), pistils including stigmas, styles and ovaries (PI), all dissected from fully open flowers, and fruit at 2, 5, 10 and 15 days post-anthesis (dpa). (B) *CcGH3* mRNA transcript accumulation was assayed by RNA blot hybridization in leaves, LF; shoots, SH; flower buds, FB; flowers, FL and fruit harvested and assayed at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 dpa. (C) Samples from (B) were assayed by RT-PCR with primers to amplify 18S rRNA added to each reaction as internal standards. (D) Accumulation of *CcGH3* transcripts in pericarp (PC), placenta (PL) and seeds (SD) of ripe fruits.

(Figure 3A and B), corresponding to fruit set, rapid cell division and cell expansion stages, periods that also coincide with active auxin synthesis during seed development (Gillaspy *et al*., 1993). *CcGH3* mRNA transcripts were first clearly evident at about 40 dpa, corresponding to the stage at which mature green fruit attained maximum size. Between 45 dpa, the onset of ripening, and 60 dpa, transcripts accumulated at much greater levels (Figure 3A, B). This analysis was confirmed using RT-PCR in which *CcGH3* had extremely low expression in fruit before 40 dpa and in leaves (Figure 3C). To determine which fruit tissues showed *CcGH3* expression, fruits were harvested at the color turning stage (45 dpa) and dissected into pericarp, placenta and seeds. *CcGH3* transcripts were undetectable in seeds, but accumulated to a very high level in pericarp and placental tissues (Figure 3D).

To eliminate the possibility that conjugated auxin synthesized in seeds was exported into the surrounding tissues resulting in induction of *CcGH3*, mature green parthenocarpic fruits were cut into 0.5 cm^2 pieces and placed on MS salt plates for periods ranging from 6 h to 10 days to induce the ripening process *in vitro*. After three days on the plate, the fruit pericarp pieces commenced the color change from green to orange. Total RNA from control and medium-incubated pericarp pieces at different time points were used for Northern analysis. In the first 48 h, *CcGH3* transcripts did not increase appreciably (Figure 4A) but gradually rose to levels comparable with those of naturally ripened fruits. Again, accumulation of

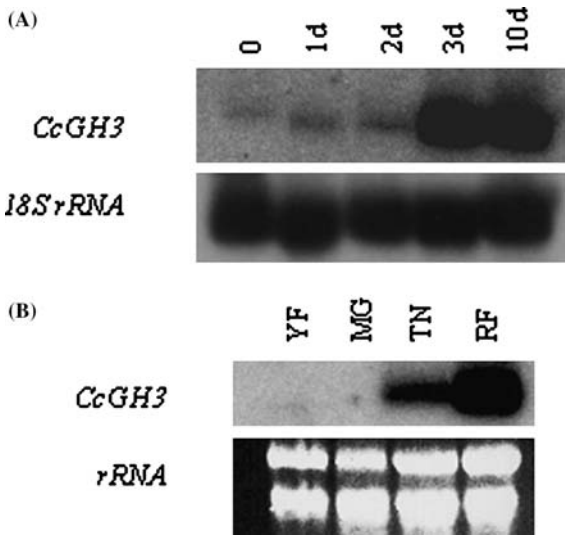

Figure 4. Accumulation of *CcGH3* transcripts in *in vitro*-ripened and parthenocarpic *C. chinense* Habanero fruit tissue. (A) Pericarp from mature green fruit (40 dpa) was excised and ripened *in vitro* for 1, 2, 3 and 10 days. (B) Parthenocarpic fruits at different developmental stages were harvested and dissected: YF, young fruit; MG, mature green fruit; TN, turning stage fruit; RF, ripe fruit.

CcGH3 transcripts in the absence of seeds was concomitant with the onset of ripening defined by color change (Figure 4B).

Distribution and concentration of auxin in pepper fruits

Because the expression of the auxin-responsive *CcGH3* gene was not detected during early stages in pepper fruit development, we examined whether or not auxin was produced early in pepper fruit development. A synthetic auxin-responsive construct *DR5*, comprised of seven repeats of the auxin-responsive element TGTCTC from the *GmGH3* promoter, was used to monitor auxin accumulation indirectly in plants. Because *DR5* functions through a single type of TGTCTC AuxRE, it provides a more specific and better characterized marker for auxin perception than natural auxin-responsive promoters, which may contain multiple AuxREs and other *cis* elements that regulate gene transcription (Ulmasov *et al*., 1997c). The activity of the auxin-responsive construct *DR5* has been highly correlated with measured free IAA levels using physicochemical methods (Sabatini *et al*., 1999; Casimiro *et al*., 2001; Friml and Palme, 2002).

To monitor the auxin distribution in pepper fruit development, the DR5:GUS reporter gene was delivered into pericarp tissues of young and ripe fruits using particle bombardment. Pericarp

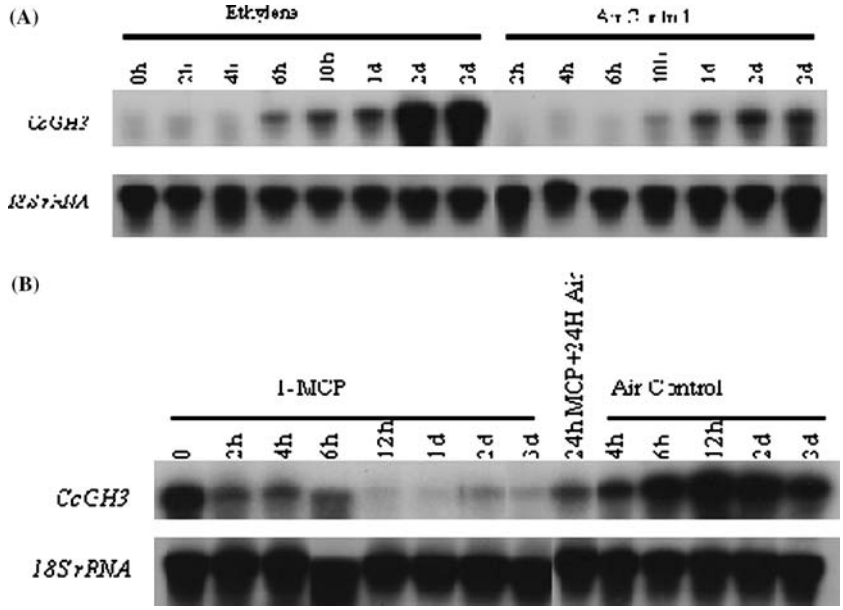

Figure 5. Expression of *CcGH3* in response to ethylene and its action inhibitor 1-MCP. (A) Pericarp of mature green pepper fruit (40 dpa) was excised, held on plates then treated with ethylene or in air only at 20 ppm (v/v) for the indicated times. (B) Ripe pericarp tissue was treated with 1-MCP, an inhibitor of ethylene action , or air control, for the indicated times. After 24 h treatment, plates were exposed to air for another 24 h. 18S RNA gene was used as the standard for equal loading of RNA samples.

samples containing the DR5:GUS construct expressed substantially more GUS than empty vector, but there was no difference between developmental stages (data not shown). Additionally, when treated with exogenous IAA, there was no change in expression of DR5:GUS. These results suggest that endogenous auxin is present at non-limiting levels in both young and ripe pepper pericarp tissue.

CcGH3 *transcripts can be induced by ethylene and inhibited by 1-MCP*

Although *CcGH3* is auxin-inducible and auxin is present in fruit, *CcGH3* expression is very low or absent until the onset of fruit ripening. In view of the importance of ethylene in fruit ripening, we tested whether ethylene affected *CcGH3* expression. To determine the profile of ethylene evolution during pepper fruit ripening, we compared six developmental stages identified by preliminary experiments: immature, mature green (MG), orange (O), and three turning stages representing degree of color change ($T_{0.25}$, $T_{0.5}$ and $T_{0.75}$). Ethylene production was only 0.07 ± 0.028 nl ethylene.g^{-1} in fruit at the IM stage, but averaged 0.65 ± 0.104 and 0.82 ± 0.36 nl ethylene.g^{-1} at the MG and O stages respectively. We investigated the turning stage fruit in more detail, separating fruit into three different classes based on the extent of color change, but no significant differences among T 0.25, T 0.5 and T 0.75 stages could be detected ($P = 0.533$). Mean ethylene production rates were 0.66 ± 0.28, 0.82 ± 0.13 and 0.86 ± 0.38 g nl g^{-1}, respectively.

For *in vitro* ripening assays, pericarp pieces from mature green fruits were placed on MS agar plates with and without ethylene, and RNA was subsequently extracted for blot analysis. Ethylene-treated pericarp began to change color slightly earlier (less than 12 h) and reached full color change 48–72 h earlier than untreated (air control) tissues. Transcript levels of *CcGH3* mRNA in untreated tissues accumulated by 10 h and gradually increased over time (Figure 5A). In contrast, *CcGH3* transcript levels increased rapidly after 6 h of ethylene application. These data indicate that exogenous ethylene promoted both ripening of pepper fruit and *CcGH3* expression.

1-MCP is an ethylene action inhibitor thought to bind irreversibly to ethylene receptors (Sisler and Serek, 1997). If *CcGH3* induction was independent of ethylene, treatment with 1-MCP should have no effect on levels of expression. Pericarp pieces from ripe fruit were treated with 1-MCP for similar time-points to those samples for ethylene treatment. In untreated tissues (air control), the transcript levels of *CcGH3* mRNA were intermediate initially and increased to a peak after 12 h (Figure 5B). In contrast, CcGH3 expression in samples treated with 1-MCP decreased substantially after 2 h and continued to decrease through time (Figure 5B). Correspondingly, 1-MCP treated pericarp pieces from mature green fruit (40 dpa) did not change color within 6 days of treatment, whereas air controls changed from green to orange after 2 days (data not shown). When pericarp pieces that had been exposed to 1-MCP for 24 h were subsequently exposed to air, transcript accumulation resumed, suggesting that there was recovery and resumption of endogenous ethylene response.

Promoter analysis

To examine the molecular basis for auxin and ethylene regulation of *CcGH3* expression we examined the promoter of *CcGH3* gene for known *cis*-acting regulatory motifs (Higo *et al.*, 1999). Sequence analysis of the 1310 bp 5′ to the *CcGH3* transcription start point identified a TATA box at –28 (Figure 6). The *CcGH3* promoter does not

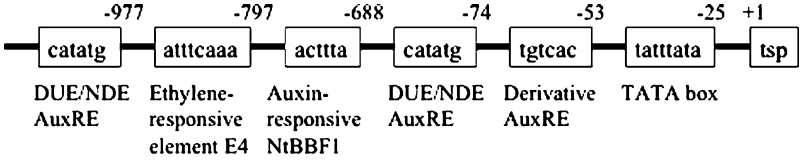

Figure 6. Structure of the promoter region of the *CcGH3* gene. The putative transcription start point is indicated (tsp, +1) in this 1310 nt sequence. Computer-deduced putative *cis* elements are indicated with the accompanying recognition sequence: DUE/NDE auxin-responsive element; ethylene-responsive element E4; auxin-responsive *NtBBF1*; derivative auxin-responsive element; TATA indicates the putative TATA box.

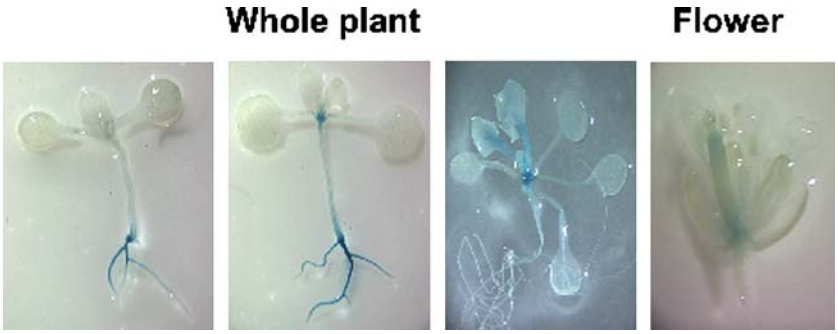

Figure 7. Histochemical analysis of GUS expression in transgenic *Arabidopsis* plants with *GH3* promoter-GUS fusion constructs.

have the typical TGTCTC auxin-responsive sequence found in the promoters of the primary auxin-responsive genes (Liu *et al*., 1994; Hagen and Guilfoyle, 2002), but the derivative TGTCAC element occurs at –57 bp. Furthermore, two CATATG elements appear at −78 and −792 bp, respectively, similar to the second auxin-response region upstream of the DUE/NDE element in the SAUR15A promoter (Xu *et al*., 1997). Finally, a NtBBF1 binding site, ACTTTA, that is required for tissue-specific expression of the Dof protein from tobacco and for auxin induction in *Agrobacterium rhizogenes* rolB gene (Baumann *et al*., 1999) was noted.

The ethylene-responsive element ATTTCAAA, was also detected. This element has been previously defined in the tomato E4 and carnation GST1 promoters (Montgomery *et al*., 1993). Taken together, this analysis reveals that several sequences known to regulate gene expression in response to both ethylene and auxin were present in the promoter of the *CcGH3* gene.

GUS reporter gene constructs in Arabidopsis

To study the expression of pepper *GH3* histochemically, we developed transgenic *Arabidopsis* plants containing a *GH3* promoter and GUS fusion gene. We tested more than 20 independent transgenic lines and the GUS activity patterns were similar for all transgenic plants. At the seedling stage, GUS activity was detected in roots at the branching points of lateral roots and root tips and was also seen at the point of leaf branching from the meristem (Figure 7). In *Arabidopsis* flowers, GUS activity was detected in the stigma and the ovary.

Over-expression of CcGH3 *in tomato and* Arabidopsis

In tomato, the *GH3* expression was substantial in flowers, but no gene expression was detected by Northern blot analysis in tomato fruit (Figure 8A).

To test the effects of *CcGH3* over-expression, transgenic tomato plants were made by introducing the full-length *CcGH3* cDNA under the control of 35S promoter. A total of seven independent kanamycin resistant putative transgenic plants were obtained. Northern blot analysis of transgenic plants showed that with one exception, all of the transgenic plants over-expressed *GH3* gene in leaf tissues (Figure 8B). Age-matched mature green fruits from control and two independent GH3 transgenic lines (8-1, 8-4) were treated with ethylene to investigate the affect of over-expression of GH3 on ripening. Both transformed lines ripened 48–72 h earlier than control fruit (Figure 8C).

To investigate whether the pepper *GH3, CcGH3*, could function in *Arabidopsis*, a *35S:CcGH3* construct was transformed into the Columbia ecotype. The over-expression of *CcGH3* resulted in a similar phenotype as previously seen in *Arabidopsis* plants over-expressing the *Arabidopsis* GH3, YDK1 (Takase *et al*., 2004). Five transgenic lines out of 14 tested showed significantly reduced hypocotyl lengths as compared to wild-type controls (data not shown).

Discussion

We have isolated and characterized an early/primary auxin-response gene, *CcGH3* from ripe pepper fruit. *CcGH3* does not have any previously

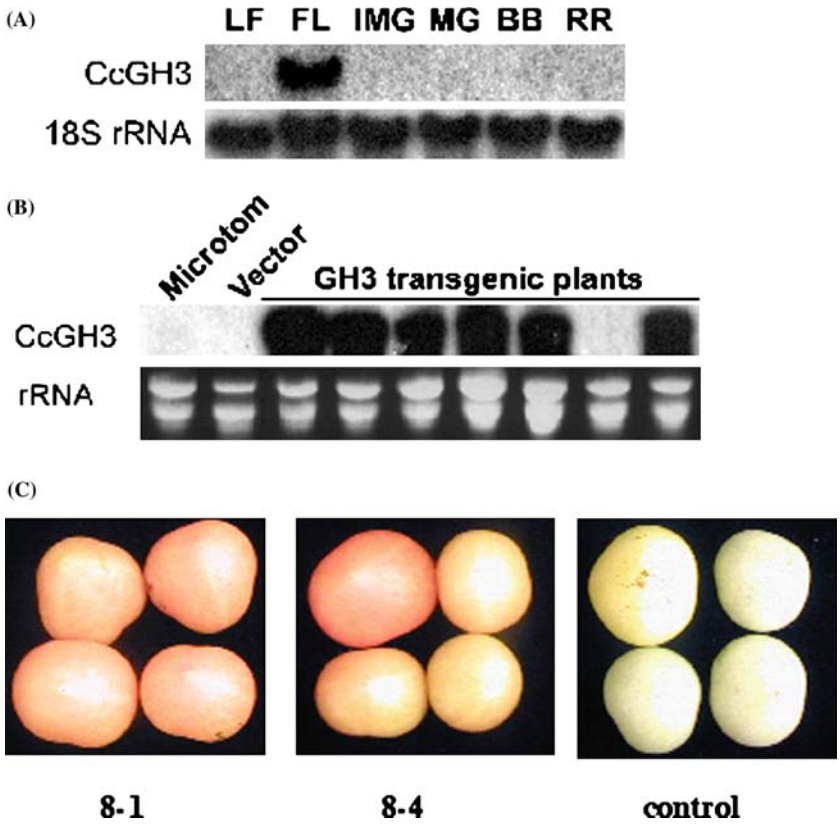

Figure 8. CcGH3 expression in tomato. (A) Accumulation of GH3 transcript in different tissues of wild type tomato: LF, leaves; FL, flower; IMG, immature green stage fruit; MG, mature green stage fruit; BB, breaker stage fruit; RR, red ripe stage fruit. 18S ribosomal RNA expression was used as an internal control. (B) GH3 gene expression in leaves of transgenic plants (35S:CcGH3) and wild type tomato. *Solanum lycopersicum* cv. Microtom used for transformation; vector, transgenic plant harboring empty vector. (C) Age-matched fruit from control plants and two independent transformed lines over-expressing CcGH3 treated with exogenous ethylene for 8 h. Control, wild-type Microtom control fruit; 8-1 and 8-4, two independently transformed 35S:CcGH3 transgenic plants.

identified motifs or domains in the deduced amino acid sequence of the open reading frame and is predicted to be cytoplasmic (Nakai and Horton, 1999), characteristics that are consistent with reports for other members of the *GH3* gene family (Roux and Perrot-Rechenmann, 1997; Hsieh *et al*., 2000; Nakazawa *et al*., 2001). In the absence of exogenous auxin, *CcGH3* mRNA transcripts were very low or undetectable in seedlings, but were induced upon application of exogenous auxin in a dose-dependent manner. Promoter analysis indicated that *CcGH3* did not have the typical TGTCTC sequence found in both simple and composite auxin-response elements in many early/primary auxin-response gene promoters (Ulmasov *et al*., 1997a, c). However, it has two copies of the novel auxin-responsive element, CATATG, which is also found in the promoter of SAUR15A. Two auxin-responsive regions have been reported in the SAUR15A promoter, both of which include the six-base pair sequence, CATATG, which is important for auxin response (Li *et al*., 1994; Hagen and Guilfoyle, 2002). Detailed promoter deletion and mutagenesis analysis will be required to define the role this element plays with respect to promoter function in pepper.

Pepper fruit ripening (*Capsicum* spp.) is generally classified as non-climacteric (Watkins, 2002), although varying patterns of ethylene and carbon dioxide production are observed, and there have been reports that hot pepper is climacteric (Gross *et al*., 1986; Villavicencio *et al*., 1999). Our results indicate that ethylene is evolved during pepper fruit ripening, albeit at relatively low levels, and that the highest levels are observed at developmental stages undergoing rapid ripening. Furthermore, color

change was delayed by treatment with the inhibitor of ethylene perception, 1-MCP. Control of ripening in non-climacteric fruit is less well understood than for climacteric fruit, although there is evidence that they share some regulatory features. The expression of *LeMADS-RIN* in strawberry fruit, which is involved in ethylene-independent events in tomato fruit ripening, suggest a common function in both ripening types (Moore *et al*., 2002).

In contrast to increasing levels of ethylene seen at the onset of fruit ripening, and the present observation of increased expression of auxin-responsive CcGH3 at late stages of fruit development, auxin levels are usually high early in fruit development and then decrease prior to ripening in both climacteric and non-climacteric fruits (Given *et al*., 1988; Jones *et al*., 2002; Balbi and Lomax, 2003; Ozga and Reinecke, 2003). In strawberry, ripening is strongly influenced by auxin levels; a decline in these levels in the receptacle, possibly due to the cessation of auxin transport from the achenes, is strongly associated with initiation of ripening (Given *et al*., 1988).

In the present study, however, *in vitro* expression of *DR5:GUS* indicated that while auxin was present in young, developing pepper fruit, it was also present in ripe fruit. Moreover, *CcGH3* was shown to contain auxin-responsive elements in its promoter and its expression was up-regulated by exogenous auxin. Paradoxically, *CcGH3* transcript was undetectable in the reproductive organs early in fruit development or in young developing fruit when auxin concentrations are expected to be highest, and was strongly induced at about 45 dpa when auxin is expected to decline. These data indicate that auxin itself may not be sufficient to induce CcGH3 expression *in vivo*. Other factors, such as ethylene, produced by the ripening fruits, may be required, or *CcGH3* expression may regulated by combinations of stimuli, e.g. auxin and ethylene, in different tissues at different developmental stages.

Studies indicate that regulation by auxin is complex, as it involves antagonistic processes and interactions with numerous regulatory systems (Hagen and Guilfoyle 2002; Swarup *et al*., 2002; Berleth *et al*., 2004). Among the interactions, auxin has been shown to stimulate ethylene production in numerous species and tissue systems (Bui and O'Neill, 1998; Shiomi *et al*., 1998; Li *et al*., 2003) In non-climacteric strawberry fruit, IAA has been shown to interact with a system for feedback inhibition of ethylene synthesis such that release of this inhibitory effect enhances ethylene synthesis (Tian *et al*., 1997).

In the present study, we used an *in vitro* pericarp ripening assay and treatment of pericarp pieces with ethylene and 1-MCP to avoid potential confounding effects associated with transport of auxins generated by seeds. Tissue-specific expression of *CcGH3* in ripe fruit indicated that it was expressed at highest levels in pericarp and placenta tissues. Similar expression was observed in parthenocarpic fruit and *in vitro*-ripened pericarp tissue. Pericarp pieces were responsive to ethylene treatment, evident by color change from green to red, consistent with previous observations (Mao and Motsenbocker, 2002). Color change of pericarp tissue treated *in vitro* was consistently inhibited by 1-MCP treatment with a concomitant drop in *CcGH3* transcript level.

Increased expression of *CcGH3* transcript in pepper was concomitant with the onset of fruit ripening, and associated with low but gradually increasing ethylene concentrations. Collectively, the current findings indicate that ethylene may be a critical factor in the regulation of *CcGH3*, possibly in concert with auxin and tissue-specific factors. While the mechanisms that provide for crosstalk between these pathways remain obscure, there is some evidence of their points of regulatory contact. In melon, a gene encoding 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) is auxin-responsive and has nucleotide sequences resembling the auxin-responsive elements (AuxRD) D1 and D4 (the TGTCTC element) in the *GH3* gene from soybean in the 5′-flanking region (Ishiki *et al*., 2000). In tomato fruit, several auxin up-regulated genes of the Aux/IAA family are differentially regulated by ethylene – some up- and some down-regulated (Jones *et al*., 2002). Among them, DR3 is strongly up-regulated by ethylene and auxin, and its expression is maximal at late stages of fruit development.

Because pepper is not easily transformed, *CcGH3* was over-expressed in tomato to gain insight into the possible function of this gene in fruit ripening. Shortened hypocotyls were observed in a number of transgenic lines consistent with previous analyses of mutants at loci encoding *GH3*-like genes (Hsieh *et al*., 2000). Furthermore, we recovered lines showing altered ripening profiles. Mature green fruit from tomato plants over-expressing *CcGH3* ripened faster than control

plants when exposed to exogenous ethylene, perhaps indicating a role for *CcGH3* in ethylene perception or signaling. Our observations are consistent with a mechanism by which an increase in *CcGH3* transcript correlates with an increase in ethylene responsiveness. This phenotype, taken with the expression profile of CcGH3 in pepper and the low levels of ethylene present during pepper fruit ripening may point to a role for CcGH3 as an amplifier of ethylene response. This study extends the role of GH3-like genes to fruit ripening in plants, and suggests CcGH3 as an interesting candidate in the dissection of the roles of auxin and ethylene in non-climacteric fruit ripening.

Acknowledgements

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