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Screening and Selection of High Carotenoid Producing in Vitro Tomato Cell Culture Lines for [^{13}C]-Carotenoid Production

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Abstract

Isotopically labeled tomato carotenoids, phytoene, phytofluene, and lycopene, are needed for mammalian bioavailability and metabolism research but are currently commercially unavailable. The goals of this work were to establish and screen multiple in vitro tomato cell lines for carotenoid production, test the best producers with or without the bleaching herbicides, norflurazon and 2-(4-chlorophenyl-thio)-triethylamine (CPTA), and to use the greatest carotenoid accumulator for in vitro ^{13}C-labeling. Different *Solanum lycopersicum* allelic variants for high lycopene and varying herbicide treatments were compared for carotenoid accumulation in callus and suspension culture, and cell suspension cultures of the *hp*-1 line were chosen for isotopic labeling. When grown with [U]-^{13}C-glucose and treated with CPTA, *hp-1* suspensions yielded highly enriched ^{13}C-lycopene with 45% of lycopene in the M+40 form and 88% in the M+35 to M +40 isotopomer range. To the authors' knowledge this is the first report of highly enriched ^{13}C-carotenoid production from in vitro plant cell culture.

Keywords

Solanum lycopersicum; *Solanum pimpinellifolium*; plant cell culture; lycopene; phytoene; metabolic tracing; isotopic labeling; carotenoids; tomato; nutrition; metabolism

1. INTRODUCTION

Frequent tomato consumption is believed to contribute to a balanced diet as well as reduce chronic disease risk, most notably prostate cancer and cardiovascular disease (1). Tomatoes are a rich source of vitamins and minerals, as well as pro-vitamin A and nonpro-vitamin A carotenoids. Carotenoids are lipophilic tetraterpenes with conjugated double bonds, which are responsible for the bright orange, yellow, and red colors seen in nature (2). Raw, red

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tomatoes contain substantial amounts of the red carotenoid lycopene (LYC), the colorless carotenoids phytoene (PE) and phytofluene (PF) (9270, 1860, and 820 μg/100 g, respectively), and smaller amounts of cyclic carotenoids and xanthophylls such as β-carotene and lutein (230 and 80 μg/100 g, respectively) (3), although these concentrations can vary substantially by ripeness and cultivar.

The health protective effects of tomatoes are widely believed to be due to carotenoid bioactivity; however, the exact mechanisms responsible are debatable (1, 4). An array of LYC metabolic and oxidative products have been detected in both tomato-based foods and human serum, but it remains unclear whether these products are endogenously produced or absorbed from the food products (5). PE and PF are absorbed from foods and are present in human plasma after 4 weeks of tomato juice consumption (6) and also show different bioaccumulation patterns than LYC in rats in response to a 4wk tomato powder feeding regimen (7). The overarching questions regarding carotenoid bioactivity are (A) are intact carotenoids or metabolic and/or oxidative products of these carotenoids bioactive? (B) Is LYC solely responsible for bioactivity, or are the other colorless carotenoids, PE and PF, also bioactive? In order to answer these questions, biolabeled carotenoids are necessary for animal and human feeding studies to trace the differential absorption and metabolism of these prominent tomato carotenoids.

Plant cell culture is an efficient tool for producing labeled plant secondary metabolites. In vitro plant methodologies allow for rigorously controlled growing conditions, reliable biochemical responses, short growth periods, targeted production of desired phytochemicals, simplified extraction, and directed utilization of costly isotopically labeled carbohydrates (8). In particular, this approach is well suited for labeling secondary metabolites that are found in only plant fruit tissues such as tomatoes and berries, where whole plant labeling via enriched water or carbon dioxide would be wasteful and time-consuming. Whole plant or intrinsic labeling, however, is well suited for plants that can be grown quickly, and the *entire* plant can be used as a source of the desired phytochemical (for an example of intrinsic labeling, see ref 9). Previously, tomato carotenoids, red clover, and kudzu isoflavones, and grape and berry polyphenols have been successfully radiolabeled (i.e., ^{14}C) using in vitro plant cell cultures for utilization in animal phytochemical metabolism research (8, 10–14), and grape flavonoids have been ^{13}C-labeled (15, 16). However, this technology, to the authors' knowledge, has not yet been applied to the production of ^{13}C-labeled carotenoids for mammalian metabolic research.

Two general approaches exist for the efficient production of secondary metabolites from plant cell cultures. First, plants known to hyper-produce a desired phytochemical can be used to derive an in vitro cell line, and that cell line can be evaluated for its phytochemical production profile. Second, elicitors or enzyme modulators can be used to induce or enhance secondary metabolite production and accumulation in in vitro cultures (17). LYC, PE, and PF accumulation in tomato cell culture can be enhanced by treating with the bleaching herbicides norflurazon and/or 2-(4-chlorophenyl-thio)triethylamine (CPTA) (10, 11). Norflurazon inhibits phytoene desaturase, leading to an accumulation of PE and PF, while CPTA inhibits lycopene cyclase, leading to an increase in LYC accumulation (10).

The goal of the following work was to establish tomato cell lines for the efficient production of ^{13}C-carotenoids found in tomato fruit for nutritional research. A screening program was implemented to identify high LYC and high PE and PF accumulating cell lines then their carotenoid production in response to herbicide treatments tested (Figure 1A and B). The *ghost* (*gh*) phenotype tomato was used to derive plant cell cultures to investigate their potential to produce PE and PF (Figure 1A). The *gh* plant is deficient in plastid terminal oxidase (PTOX), which is a plastoquinone-O_2 oxidoreductase that likely serves as a

necessary cofactor for carotenoid dehydrogenases. The lack of PTOX, therefore, interrupts efficient carotenogenesis (18). There are nine genetic loci known within tomatoes that control fruit pigmentation; therefore, several putative high LYC producing tomatoes were selected to be scanned for in vitro LYC production. *Solanum lycopersicum* plants carrying *hp-1*, *hp-2^dg*, *B^og*, and *B^og^c* alleles in these loci previously reported to have enhanced LYC accumulation were scanned and compared to the previously established VFNT cherry tomato cell line, a line used for the production of radiolabeled carotenoids and in vitro carotenogenesis studies (10, 11, 19). An additional wild species of tomato, *Solanum pimpinellifolium*, was also selected for its elevated LYC accumulation (19). The best performing high LYC cell line was then grown with [U]-^{13}C-glucose to produce ^{13}C-LYC, which was purified and analyzed for isotopic enrichment (Figure 1B).

2. MATERIALS AND METHODS

2.1. Ghost Plant Material and Culture Initiation

Seeds from *gh*/+ tomato (*Solanum lycopersicum* cv. Mill, donated by Wendy White, Iowa State University) were germinated under very low light (9 μE $m^{-1}s^{-1}$), and after 5 days, low irradiance was maintained (~80 μE $m^{-1}s^{-1}$) to allow for viable variegated *gh* (*gh/gh*) seedlings since higher light intensities cause a lethal white-leafed phenotype (20). The seedling genotype was identified by the presence of either purple shoots and variegated or white leaves (*gh/gh*; *gh*), or green leaves (+/+ or *gh*/+; wild-type) (20). Stem and leaf segments were collected at 4 weeks, and flower buds at 11 weeks from greenhouse-grown plants for disinfestation by dipping in 70% aqueous ethanol, immersing in 7.5% sodium hypochlorite with one drop of Tween-20 (Sigma-Aldrich, St. Louis, MO) for 15 min, and rinsing with sterile distilled water for 5min. Explants were placed onto 40 mL of sterile, agar-solidified Murashige and Skoog salt-based media containing the growth regulators 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine (maintenance media) for tomato callus induction as previously described (11) in GA-7 cubes (Magenta Corporation, Chicago, Illinois). During callus induction, *gh* stem and leaf-derived calluses were transferred to fresh solidified media after 35 days, then subsequently every 21 days. To promote healthy callus growth, the *gh* flower bud-derived callus was transferred to fresh solidified media after 23 days, 12 days later, 9 days later, and subsequently every 21 days. Leaf segments and flower buds from wild-type plants were transferred to fresh solidified media after 41 days and subsequently every 21 days. Cell suspension cultures were initiated with 2 g of friable callus added to the same callus induction media as mentioned above, formulated without agar. Cell suspension cultures were subcultured every 2 weeks by the transfer of 6 mL aliquots of established cultures (2 mL of packed cells with 4 mL of spent media) to fresh maintenance media.

2.2. Carotenoid Evaluation of Different Ghost Genotypes in Callus and Cell Suspension Cultures

For carotenoid analysis, proliferating *gh* and wild-type callus cultures were initiated with 2 g of callus/40 mL of solid maintenance media in GA-7 cubes (n = 4–6) and were harvested after 3 weeks. Harvested samples were stored at −80 °C until carotenoid extraction and HPLC analysis. Carotenoid yield was evaluated for *gh* cell suspension cultures grown in either liquid maintenance media or the previously described carotenoid production media containing the growth regulators indole-3-acetic acid (5 mg/L) and *all-trans*-zeatin (2 mg/L) (11). Suspension cultures were continuously maintained at 2 week subculture intervals by inoculating either 40 mL of maintenance media in 125 mL flasks with 6 mL of established cultures or 80 mL of carotenoid production medium in 250 mL flasks inoculated with 12 mL of established cultures (4 mL of packed cells and 8 mL of spent media) and were grown (n = 5/media formulation) for one 2 week growth period and then harvested.

2.3. High LYC Cell Line Plant Material and Culture Initiation

Seeds of *Solanum pimpinellifolium* (LA0376) and *Solanum lycopersicum* cultivars [‘Ailsa Craig’ *hp-1* (LA3538), ‘Manapal’ *hp-2^dg* (LA2451), ‘Ailsa Craig’ *B^og* (LA3311), wild-type ‘Ailsa Craig’ (LA2838A), and wild-type ‘Manapal’ (LA3007)] were obtained from the Tomato Genetics Research Center, UC-Davis, CA, USA. *Solanum lycopersicum* ‘Suncoast’ $B^{\wedge}og^{c}$ seeds were obtained as a gift from Jay Scott and ‘VFNT cherry’ seeds from Betty Ishida. Germinated seeds were allowed to reach maturity in a greenhouse. Flower buds and leaves were used to generate callus cultures as described above. Callus was subcultured every 4 weeks for at least 2 growth cycles until friable calluses developed. Transfer of 2 g of callus to 40 mL of maintenance media initiated liquid suspension cultures. Cell suspensions were subcultured every 2 weeks as described above.

2.4. Herbicide Treatment Experiments

Cultures were either treated aseptically on day 1 with filter-sterilized aqueous CPTA (donated by Betty K. Ishida, USDA ARS, St. Albany, CA) (0.0745 g/L media) (10) and/or on day 7 with filter-sterilized norflurazon (Syngenta, Greensboro, NC) dissolved in dimethyl sulfoxide (0.06 mg/80 mL media) as previously published (11). To investigate the potential for increasing carotenoid accumulation in *gh* and wild-type cell suspension cultures, either norflurazon or CPTA was added to *gh* and wild-type cultures. Herbicides were provided to *gh* and wild-type cell suspension cultures in 80 mL of the carotenoid production medium. High LYC tomato cell lines were also treated with CPTA to stimulate LYC accumulation either by aseptic addition to freshly autoclaved callus carotenoid production media before solidification or to cell suspension cultures in liquid carotenoid production media. Callus cultures were grown with or without CPTA in the solid media for one 4 week growth cycle and suspensions for 2 weeks. The *hp-1* tomato cell line was treated with CPTA and/or norflurazon, as specified, to investigate the potential to manipulate the carotenoid accumulation profile.

2.5. Callus and Cell Suspension Culture Harvest

Callus cultures were harvested by careful separation of the callus from solid media using metal spatulas and weighed, and fresh mass was recorded. Labeled and nonlabeled cell suspension cultures were harvested using Whatman #4 filters with a Büuchner funnel over a vacuum. Filtration was ended, and cells were collected and weighed when no liquid was expressed from the funnel for 30 s. All collected culture samples were stored under argon in containers, capped, sealed with Parafilm, and stored at −80 °C until further analysis. Samples (~5 g) of each ^{13}C labeled culture were reserved for further carotenoid and ^{13}C mass spectrometry analysis, and the remaining cells were combined for carotenoid extraction and preparatory separation for future mammalian metabolic studies.

2.6. HPLC Carotenoid Evaluation

Callus and suspension cultures were analyzed for carotenoid content by extracting ~0.25 g of cells using a carotenoid extraction and high pressure liquid chromatography–photodiode array (HPLC-PDA) analysis protocol previously published (11). General precautions for work with carotenoids were taken to prevent degradation and artifact formation during the extraction and analytical process (21). Carotenoids were identified and quantified by external standard curves using authentic LYC (isolated from DSM Redivivo Beadlets donated by DSM, Heerlen, Netherlands) and PE and PF standards (gifts from Hansgeorg Ernst, BASF, Ludwigshafen, Germany).

2.7. Selection of the Highest LYC Accumulating Cell Line

Callus cultures of mutant high LYC lines, corresponding wild-type cell lines, and the 'VFNT cherry' cell line were evaluated for LYC accumulation during 3 trials with 2 reps per trial. Each rep consisted of 3 g of callus inoculum placed onto agar-solidified carotenoid production medium with or without CPTA. Mutant high LYC line CPTA-treated cultures were compared to corresponding CPTA-treated wild-type lines to verify that the mutation was responsible for LYC accumulation differences. The highest LYC accumulators and available corresponding wild-type callus cell lines were then used to initiate cell suspension cultures by transferring 2 g of friable callus cultures to liquid maintenance medium. After 8.5, 10.5, and 11 months of culture adaptation to liquid medium, cultures were transferred to carotenoid production medium for one 2-week adaptation period and subsequently transferred to fresh carotenoid production medium with or without CPTA for a 2-week carotenoid production growth period, and then harvested for subsequent analysis.

2.8. Comparison of *hp-1* Carotenoid Production Grown with Sucrose or Glucose As the Carbohydrate Source

Presently, the only economical [U]-^{13}C labeled carbohydrate for addition to cell culture media is glucose; however, standard tomato growth media utilizes sucrose. To determine the effect of medium carbohydrate source on harvest mass yield and carotenoid concentration, *hp-1* cell suspension cultures were grown in liquid carotenoid production media with either 30 g/L unlabeled sucrose or unlabeled glucose for a single, 14-day adaptation growth cycle, transferred to fresh media, and treated with CPTA as specified above for a 14-day growth cycle, then harvested for analysis.

2.9. Production of ^{13}C-Labeled LYC from *hp-1* Tomato Cell Suspension Cultures

For LYC ^{13}C-biolabeling, 2 flasks of 80 mL liquid carotenoid production medium containing 30 g/L unlabeled glucose were inoculated with established cell suspension cultures for a media adaptation growth cycle. The contents of these two flasks were combined aseptically and 12 mL aliquots of the established cultures (4 mL of packed cells with 8 mL of spent media) were used to inoculate 3 flasks of fresh medium with 30 g/L [U]-^{13}C-glucose (Cambridge Isotope Laboratories, Inc., Andover, MA). The established cultures grown for the glucose adaptation growth cycle utilize glucose as an energy source; therefore, it can be estimated that the resultant ^{13}C-glucose/^{12}C-glucose ratio in the fresh ^{13}C-containing media is greater than 10:1. Other ^{12}C-containing components in the media such as growth regulators and myoinositol accounting for less than 1% of the organic content in the media are likely minor contributors to carotenoid precursors. Cultures were grown in a previously described enclosed labeling chamber at 26 °C atop a rotary shaker (160 rpm) (22). To promote LYC accumulation, cultures were treated with CPTA on day 1, grown for 14 days, harvested, and stored for carotenoid and isotope enrichment analysis.

2.10. ^{13}C-Lycopene Mass Isotopomer Analysis

Biolabeled lycopene were isolated from *hp-1* cell suspension cultures by a series of three HPLC separations. First, a previously optimized carotenoid extraction procedure for tomato cell suspension cultures was utilized (23). Carotenoid-rich extracts were first subfractionated to yield an LYC-rich fraction using a YMC C30 preparatory column (Waters, Milford, MA) with previously described conditions (11). Mobile phases in the carotenoid containing eluate fractions were evaporated using a Savant AS160 Automatic Speedvac. This fraction was subfractionated a second time to remove coeluting contaminants using a Discovery C18 (25 cm × 4.6 mm, 5 μm) (Supelco, Sigma-Aldrich, St. Louis, MO) column following a previously published method (24). Eluted LYC was collected, and one-third and final separation was performed on the same HPLC apparatus described using a high resolution

HPLC method using a YMC C30 analytical column (4.6 × 25 cm, 3 μm) to obtain pure *all-E* LYC containing minimal coeluting carotenoids or contaminants (25). LYC from this purified fraction was then analyzed using liquid chromatography–tandem mass spectrometry (LC-MS/MS) using atmospheric pressure chemical ionization (APCI) at 2877 V, 400 °C, a nebulizer pressure of 60 psi, and dry temperature of 350 °C. Purified ^{13}C-LYC was dissolved in diethyl ether and injected onto a LC-MS/MS (LC-MSD-Trap-XCT plus, Agilent Technologies, Santa Clara, CA) equipped with a Zorbax Eclipse XDB C18 column (Agilent Technologies, Santa Clara, CA; 2.1 × 50 mm, 3.5 μm). Sensitivity of the LC-MS/MS used was 1–10 ng, and higher amounts were injected to facilitate the quantification needed for mass isotopomer analysis. To quantify the proportions of different isotopomers present in the ^{13}C-LYC peak, four injections of 1000 ng of ^{13}C-LYC in 40 μL of diethyl ether were made, and data acquisition parameters were set to collect signal intensities for LYC masses on positive ion mode ranging from *m*/*z* 572.4–577.4 (M + 35 to M + 40) for injection 1, *m*/*z* 567.4–572.4 (M + 30 to M + 35) for injection 2, *m*/*z* 562.4–567.4 for injection 3 (M + 25 to M + 30), and *m*/*z* 557.4–562.4 for injection 4 (M + 20 to M + 25). Mass chromatogram peak areas generated from the resulting runs were normalized on the basis of repeated *m*/*z* channels analyzed between runs and were then quantified. Mass chromatogram peak areas for masses below 557.4 were not quantifiable; therefore, the effective range of masses evaluated was from *m*/*z* 557.4–577.4 (M + 20 to M + 40). Relative signal intensities for carotenoid mass isotopomers were compared, and the ^{13}C-enrichment profile was quantitatively determined using spectral peak areas.

2.11. Statistical Analysis

Statistically significant treatment and cell line differences for > 2 groups were determined by analysis of variance (ANOVA), and when the normality and homogeneity of variance assumptions of ANOVA were met, the posthoc Tukey's studentized range test was used to identify specific differences. When the assumptions of ANOVA were not met, the nonparametric Wilcoxon and Kruskal–Wallis tests were used to detect significant differences. When only two treatments were being compared, the two-sample *t* test was used, and when the normality and homogeneity of variance assumptions of the *t* test were met, the equal variances *t* test was used to determine significant differences. If both the normality and homogeneity of variance assumptions of the two-sample *t* test were not met, the Wilcoxon two-sample test was used. If only the homogeneity of variance assumption of the two-sample *t* test was violated, then the Cochran modified *t* test for unequal group variance was used. All statistical analyses were performed using the statistical analysis software SAS, versions 7.1 and 9.2 (SAS Institute, Inc., Cary, NC). Averages are presented with the standard error of the mean whenever possible.

3. RESULTS AND DISCUSSION

3.1. *gh* and Wild-Type Tomato Cell Cultures

3.1.1. Callus and Suspension Culture Initiation—Eighty-percent of the seeds from wild-type ($^{+}/^{+}$ or $^{+}/gh$) tomato germinated within 9 days, and of those, 10% were positively identified as *gh* mutants by the presence of variegated true leaves. Callus growth was observed within 16 days on the surface of the *gh* stem and leaf explants and within 30 days on *gh* flower bud explants. Callus growth on wild-type explants was observed within 41 days on leaves and within 20 days on flower buds.

3.1.2. Carotenoid Accumulation by Genotype and Explant Source—Wild-type and *gh* callus cultures were sampled and analyzed for carotenoid concentrations. The *gh* flower bud-derived callus culture accumulated the greatest amount of total carotenoids with PE being the major carotenoid (5.6 ± 1.8 μg/g callus) and PF, LYC, and β-carotene (Table

1) in lower concentrations. Both leaf and flower bud-derived *gh* callus cultures had greater overall carotenoid accumulation than the wild-type callus cultures, though the wild-type-derived callus cultures had greater β-carotene concentrations than the *gh* callus cultures. The phenomenon where one in vitro vegetative tissue differs biochemically from another has been seen before (26). Previous studies of in vitro 'VFNT cherry' tomato sepals, the vegetative tissue that forms a protective layer around the flower, indicated that this tissue has the potential to undergo ripening processes including LYC accumulation, swelling, and production of ethylene and its precursor, without manipulation of the media growth regulators or attachment to fruit. The results in this article support the finding that sepal tissue of the flower bud may retain a potential to change the developmental fate from vegetative tissue to fruit-like tissue, allowing ripening processes which include increased carotenogenesis (26). In that same study, it was observed that another tomato cultivar, 'Ailsa Craig', did not exhibit the same sepal ripening process, suggesting that the involvement of a genetic component for this retained the ripening potential in sepal tissue (26). In vitro explant-specific secondary metabolite profiles from callus cultures of red clover for isoflavones and of strawberry for anthocyanins have also previously been observed (12, 27). Flower bud-derived *gh* callus exhibits more ripening processes than leaf-derived calluses; however, the wild-type callus types did not show this same trend, suggesting that there may be a genotype effect on carotenoid accumulation in differentially derived callus tissues.

3.1.3. Treatment with Herbicides—A brief investigation on the impact of CPTA and norflurazon on carotenoid accumulation in *gh* and wild-type cell suspension cultures grown in carotenoid production media (one trial/herbicide treatment, $n = 2–3$ reps/treatment) showed that the *gh* cell line yielded greater harvest mass than the wild-type cell line, regardless of herbicide treatment. In the CPTA and norflurazon experiments, *gh* cell suspension cultures yielded almost 3 times more cell mass than wild-type cultures (363 vs 95 and 347 vs 90 g cells/L culture, respectively). Counter to the initial hypotheses for this study, neither of the herbicides had a substantial impact on PE or PF accumulation in *gh* cultures; however, herbicide treatments did impact carotenoid accumulation profiles in wild-type cell suspension cultures (Tables 2 and 3). These results suggest that these bleaching herbicides may cause greater total carotenoid accumulation in wild-type cultures by causing a lack of downstream carotenoid products necessary for feedback regulation of carotenogenesis. Since the *gh* cell line already largely lacks downstream carotenoids, the bleaching herbicides did not confer any additional stimulation of PSY or inhibition of PDS. In a previous study, mRNA levels of carotenogenic enzymes in *gh* tomato fruit were similar to the levels in wild-type fruit, suggesting that a lack of downstream carotenoid products may not increase the transcription of these enzymes (20). It may be possible that the activity of the PSY enzyme is increased in the *gh* cell line compared to that in the wild-type cell line. CPTA-treated wild-type cultures did have increased LYC accumulation compared to that in the control wild-type and CPTA-treated or control *gh* cultures (Table 2). Norflurazon-treated wild-type cultures, however, did not produce as much PE as the *gh* cultures (Table 3). On the basis of these results indicating that untreated *gh* cell suspension cultures accumulate and yield the greatest amount of PE, herbicide treatment of the *gh* cell line was not pursued further. All additional *gh* experiments focused on the untreated *gh* cell line.

3.1.4. Comparison of gh Carotenoid Production from Cultures Grown in Different Media Formulations—Three trials were performed to evaluate *gh* cell suspension culture carotenoid production and cell mass accumulation from cultures grown in carotenoid production versus maintenance medium. Cultures grown in the carotenoid production media produced significantly greater cell mass (211 ± 7.2 g/L) than the maintenance media-grown cultures (150 ± 4.1 g/L) ($p = 0.0017$). PE yield was also greater in carotenoid production media (3458 ± 612 μg/L) than from maintenance media (489 ± 84

µg/L; $p = 0.04$). However, PF yield was not significantly different between media conditions (maintenance media, 27.8 ± 2.2 µg/L; carotenoid production media, 78.7 ± 16.8 µg/L; $p = 0.09$). This experiment confirmed the hypothesis that carotenoid accumulation would be greater in cultures grown on the carotenoid production media formulation. The PE yield from *gh* cultures grown on carotenoid production media is greater than that of 'VFNT cherry' tomato cell suspension cultures treated with norflurazon (2060 µg/L), a previously established suspension culture system for PE production (10). On the basis of these findings, it can be concluded that *gh* cell suspension cultures are a superior source of PE.

3.2. High LYC Lines

3.2.1. Callus Culture Initiation—Tomato flower buds from greenhouse-grown high LYC tomato plants and their corresponding wild-type plants were explanted to agar-solidified callus induction media. Callus was observed on all explants within a 1–4 week growth period, and proliferating, friable callus was obtained within 2–3 growth cycles of the 'Ailsa Craig' wild-type and *hp-1*, 'Manapal' wild-type and *hp-2*^{dg}, 'Suncoast' *B^og*^{c}, *L. pimpinellifolium*, and 'VFNT cherry'. 'Ailsa Craig' *B^og* did not adapt well to the callus induction media and was not included in subsequent studies.

3.2.2. Selection of the Highest LYC Accumulator—Three trials examining several high LYC lines, corresponding wild-type lines, and the 'VFNT cherry' line callus cultures for LYC accumulation were harvested and analyzed. Harvest yields of CPTA and untreated 'VFNT cherry' callus cultures were significantly greater than those in other CPTA or untreated cultures, respectively (Table 4). Carotenoid concentrations were greatly increased when CPTA was added to the agar-solidified carotenoid production medium (Table 5). In untreated cultures, there was no cultivar or genotype effect on combined PE, PF, β-carotene, and LYC accumulation. Alternatively, when CPTA was present in the media the *hp-1* cell line had a significantly greater combined acyclic carotenoid (PE, PF, and LYC) concentration (45.8 ± 13.1 µg/g cells) than the other cell lines including its corresponding wild-type (27.2 ± 4.5 µg/g cells), suggesting that the *hp-1* genotype is responsible for the enhanced carotenoid accumulation. All cell cultures treated with CPTA had only trace amounts of β-carotene (data not shown). The two highest carotenoid accumulators, 'Ailsa Craig' *hp-1* and 'Manapal' *hp-2* (45.8 ± 13.1 and 31.3 ± 7.1 µg/g cells, respectively), in callus culture were thus selected along with their corresponding wild-types to be transferred to solution culture and evaluated for LYC production in comparison to previously established 'VFNT cherry' cell suspension cultures.

Three trials were initially performed to evaluate 'Ailsa Craig' *hp-1* and wild-type, 'Manapal' *hp-2* and wild-type, and 'VFNT cherry' cell suspension cultures for LYC production. Over the period of the three trials, substantial changes in LYC concentration and yield were observed (Figure 2A and B). Some tomato cell lines adapted to the carotenoid production liquid medium, while other cell lines performed more poorly over the 2.5-month span in which the three trials were undertaken. The changes in harvest mass and LYC concentrations over the 2.5-month trial period were substantial, but this is common during the establishment of in vitro plant cell lines. Bourgaud and colleagues discussed this phenomenon in callus cultures previously, suggesting that in many cases erratic production of plant secondary metabolites in vitro can be linked to genetic instability (17). Genetic instability, according to their assessment, is often under-reported in publications but has been observed to take up to 16 subculture cycles, or 48 wk, to dissipate. One practical suggestion given by these authors was to use cell growth reproducibility, a polygenic characteristic, over three consecutive growth cycles as a marker for genetic stability (17). Although these authors discussed the appearance of this phenomenon in callus cultures, from the current studies, it appears that genetic instability is apparent in cell suspension

cultures and that adaptation time can be lengthy. For this reason, tomato cell line selection was based on LYC yields obtained from the final trial completed 11 months postcell suspension culture initiation. Eleven months postinitiation of cell suspension cultures, ‘Ailsa Craig’ *hp*-1 yielded the greatest amount of LYC (7.58 mg/L culture), was followed by ‘Ailsa Craig’ wild-type (5.47 mg/L culture), and was 1.6-fold greater than ‘VFNT cherry’ LYC yield (2.97 mg/L culture) (Figure 2B). ‘VFNT cherry’ cell suspension cultures produced the highest concentrations of PE with CPTA-treatment (5.22 μg/g cells) and were also the greatest accumulators of PE when untreated (0.70 μg/g cells), compared to the other cell lines (Table 6). ‘Ailsa Craig’ *hp-1* also yielded the greatest cell mass (199 g/L) at the 11-month harvest. The high cell mass production combined with high concentrations of LYC led to the selection of the ‘Ailsa Craig’ *hp-1* for subsequent herbicide treatment and ^{13}C-biolabeling studies. To ascertain genetic stability, the three subsequent herbicide trials were run consecutively, per the recommendations of Bourgaud et al. (17).

3.2.3. Treatment with Herbicides—Three consecutive trials with three repetitions per trial were harvested to simultaneously evaluate the genetic stability of and carotenoid accumulation in *hp-1* cell suspension cultures in response to different herbicide treatments. Within the control, CPTA, and norflurazon treatments, there were no significant harvest mass yield differences between trials according to the Kruskal–Wallis test (average yields were 218 ± 42 g/L, 156 ± 16 g/L, and 220 ± 40 g/L, respectively). However, there was a significant difference within the CPTA and norflurazon combination treatment group between trials ($\alpha = 0.05$) between the first (214 ± 33 g/L) and third (119 ± 3 g/L) trials, but neither was different from the second trial (183 ± 3 g/L) according to Tukey’s studentized range test. Although there were differences between the harvest yields of the CPTA and norflurazon combination treatment between trials, the remainder of the treatments had statistically consistent harvest yields and can be viewed, on the whole, as genetically stable. When harvest yields were compared between treatments for all trials, there were no significant differences, including the CPTA and norflurazon combination treatment (average 172 ± 28 g/L; other treatment averages given above). Carotenoid yields were analyzed for each trial (Figure 3). There were no significant differences in individual carotenoid concentrations by herbicide treatment; however, total carotenoid yields in herbicide-treated cultures were significantly greater than the control cultures ($\alpha = 0.05$) (Figure 3). The PE, PF, and LYC accumulation patterns were similar to, but overall greater than, the accumulation pattern previously seen in ‘VFNT cherry’ tomato cell suspension cultures treated with these herbicides (10) (3.6–5.2 mg/L culture vs 2.3–3.3 mg/L culture, respectively) (10). This result suggests that *hp-1* tomato cell suspension culture is superior to the previously established ‘VFNT cherry’ cell suspension culture system. The untreated *gh* tomato cell line, when grown on carotenoid production media, also produced substantially more PE than previously published values for the norflurazon-treated ‘VFNT cherry’ line, but had a lesser PE accumulation than the norflurazon-treated *hp-1* cell line. On the basis of these findings, the *hp-1* cell line treated with CPTA was selected for ^{13}C-LYC labeling.

3.3. Tomato Cell Suspension Culture ^{13}C-Carotenoid Biolabeling

3.3.1. Comparison of Carotenoid Production in hp-1 Cell Suspension Cultures Grown with Sucrose or Glucose As the Carbohydrate Source—Carbohydrate source in the tomato cell culture liquid media did not have a significant impact on harvest mass yield (glucose, 199 ± 19 g/L; sucrose, 206 ± 2 g/L). Neither total carotenoid yield (glucose, 6.22 ± 0.34 mg/L; sucrose, 5.81 ± 1.17 mg/L) nor individual carotenoid proportions significantly varied by carbohydrate source (data not shown).

3.3.2. ^{13}C-Labeled Carotenoid Yields from hp-1 Cell Cultures—Harvest mass yields for *hp-1* cell cultures grown with uniformly labeled ^{13}C-glucose as the primary

carbon source (198 ± 3.6 g/L) were very similar to yields from cultures grown with unlabeled glucose. Carotenoid yields were, however, slightly lower than those seen in the herbicide and carbohydrate source experiments (PE, 0.313 ± 0.013; PF, 0.12 ± 0.01; LYC, 2.45 ± 0.11 mg/L). After three HPLC purification steps of the plant cell culture carotenoid extract, purified LYC was obtained (Figure 4). LYC was analyzed and purified for mass isotopomer analysis, and the results are shown in Figure 5. In vitro labeling does not yield 100% uniformly labeled LYC but rather a range of isotopomers (*m*/*z* = 557–577), which represent the M + 20 to +40 isotopomer masses. The uniformly labeled isotopomer (*m*/*z* = 577, M + 40) was the most prominent isotopomer constituent (45%) of the labeled LYC fraction, and 88% of the LYC molecules were present as M + 35 to +40 masses. Production of a distribution of β-carotene and LYC mass isotopomers has been previously reported in other biological systems (28–30). By providing uniformly labeled glucose as the primary source of carbon in this in vitro system, the labeled LYC produced from this experiment had a much greater proportion of uniformly labeled (*m*/*z* = 577) LYC than what has been seen in these other reports of deuterium enriched β-carotene and LYC. In this experiment, the unenriched isotopomer (*m*/*z* = 537) was not detected in purified ^{13}C-LYC isolated from *hp-1* cell cultures. In conclusion, a strategic screening and selection process for carotenoid accumulation in multiple in vitro tomato cell lines noted for their elevated in vivo carotenoid accumulation was used to develop a production system for isotopically labeled carotenoids for utilization in human and animal metabolic tracing studies. To the authors' knowledge, this is the first report of the production of highly enriched ^{13}C-carotenoids from a plant cell culture system for dietary research.

Future work should focus on stimulating PF accumulation in tomato cell suspension cultures. Additionally, even higher uniform enrichments of tomato cell line-derived ^{13}C-carotenoids may be achieved by sequential growth cycles in ^{13}C-glucose containing media. Future research will focus on the utilization of this highly enriched ^{13}C-LYC as well as the production of ^{13}CPE and PF from norflurazon-treated *hp-1* cell suspension cultures for mammalian cell culture and human studies. The advances made in this work provide the opportunity for greater elucidation of mammalian carotenoid metabolism as well as underlying mechanisms of bioactivity.

ABBREVIATIONS USED

LYC	lycopene
PE	phytoene
PF	phytofluene
CPTA	2-(4-chlorophenyl-thio)triethylamine
PTOX	plastid terminal oxidase
HPLC-PDA	high pressure liquid chromatography–photodiode array
BHT	butylated hydroxytoluene

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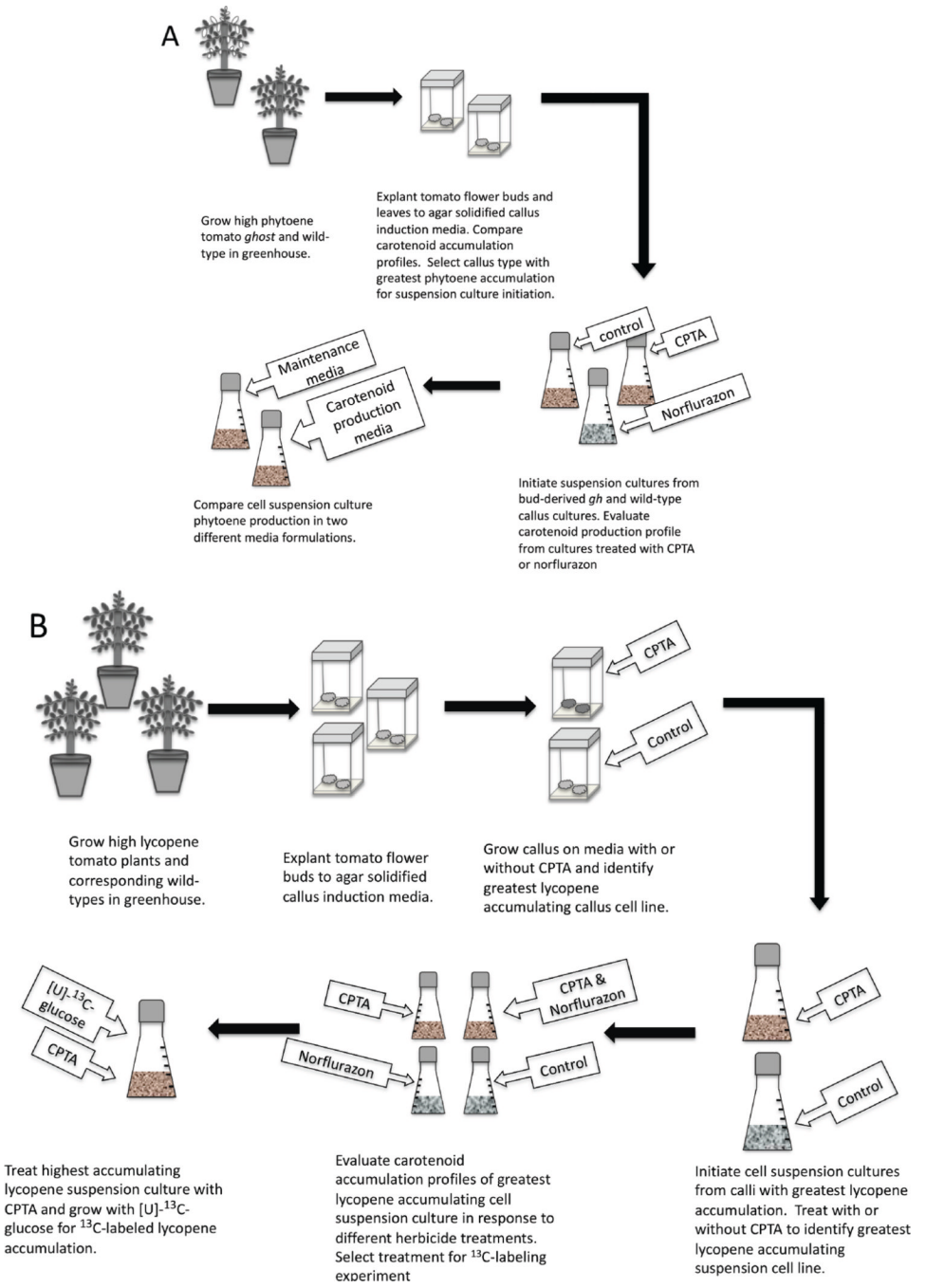

Figure 1.

Simplified study design for the derivation and selection of tomato cell lines for (**A**) phytoene production from the *ghost* tomato cell line and (**B**) lycopene, phytoene, and phytofluene production from a high lycopene tomato cell line.

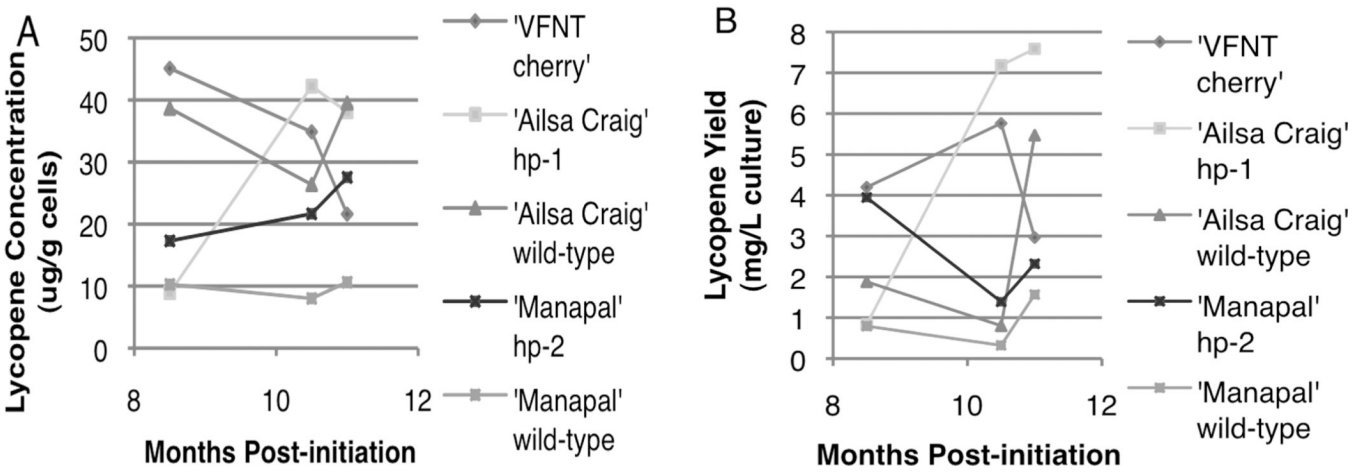

Figure 2.

Lycopene concentrations (**A**) and yields (**B**) from different tomato cell line suspension cultures treated with CPTA for a 14 day growth cycle and harvested approximately 8.5, 10.5, or 11 months postinitiation of cell suspension cultures from callus cultures. Each point represents the average of two samples.

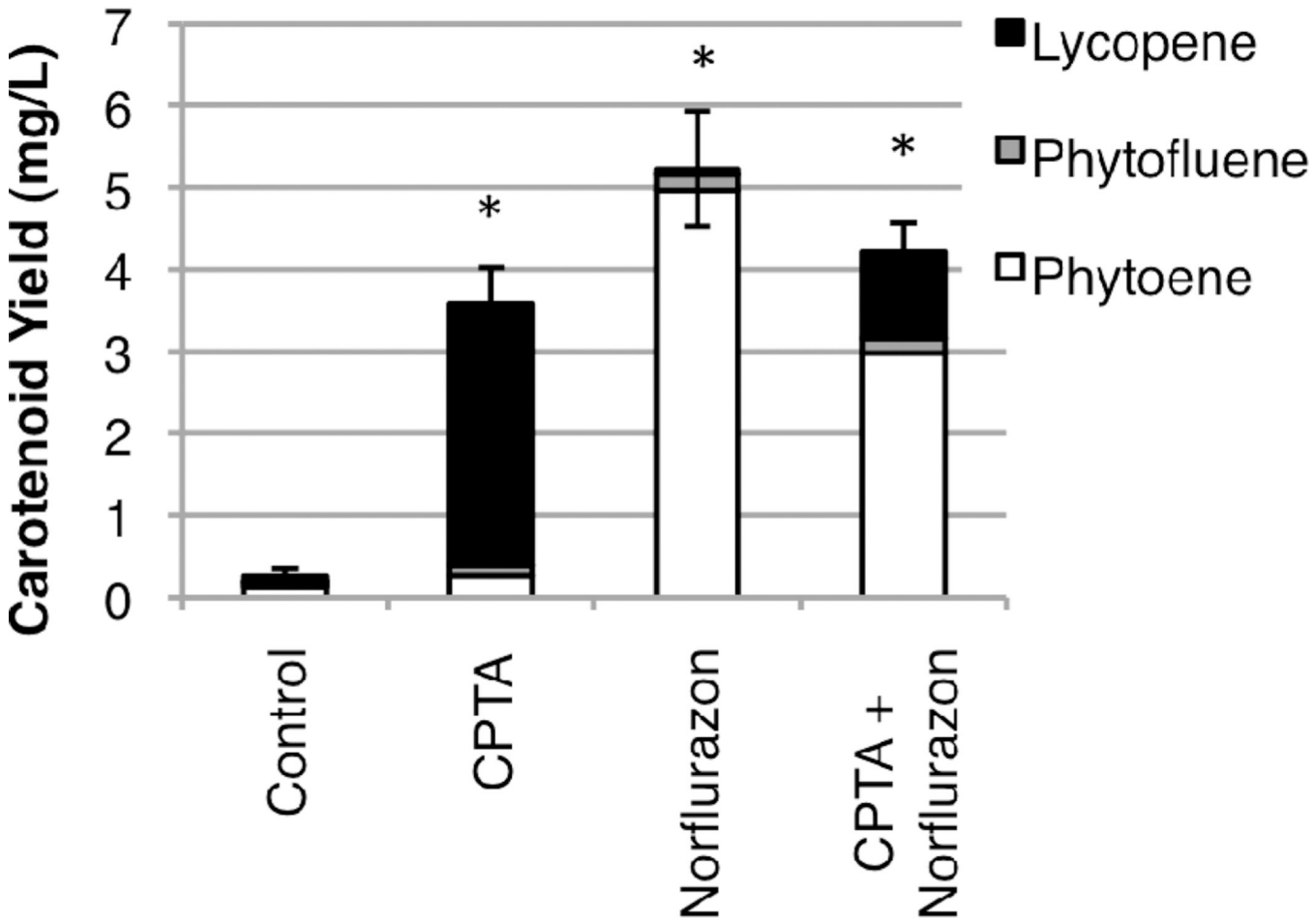

Figure 3.
Combined carotenoid (LYC, PF, and PE) yields from *hp-1* tomato cell suspension cultures treated with different herbicides ($n = 3$ trials). Error bars represent the SEM of combined carotenoids. Significantly different combined carotenoid yields ($\alpha = 0.05$) detected by Tukey's studentized range test compared to the control are noted with an asterisk (*).

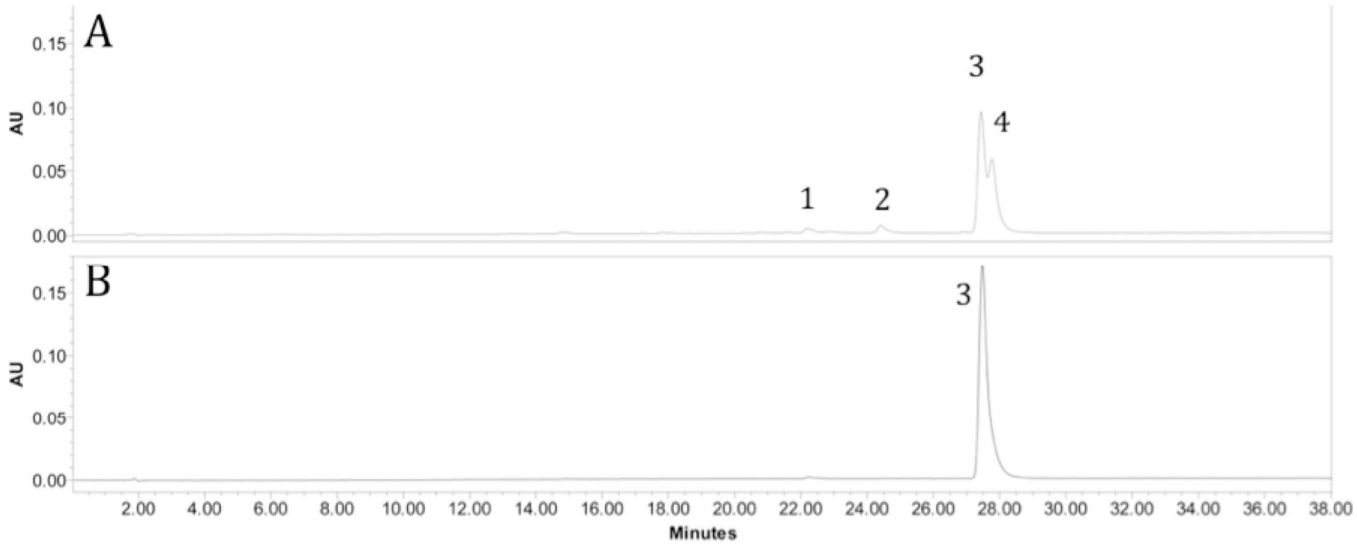

Figure 4.
HPLC chromatograms (λ = 472 nm) of (**A**) the LYC analytical standard with *all-E* (peak 3) and 5-*Z* (peak 4) lycopene isomers eluting at 28 min with other preceding *Z*-isomers (RT = 22.5 and 24.5 min; peaks 1 and 2) and (**B**) purified ^{13}C-*all-E* LYC from an *hp-1* tomato cell suspension culture.

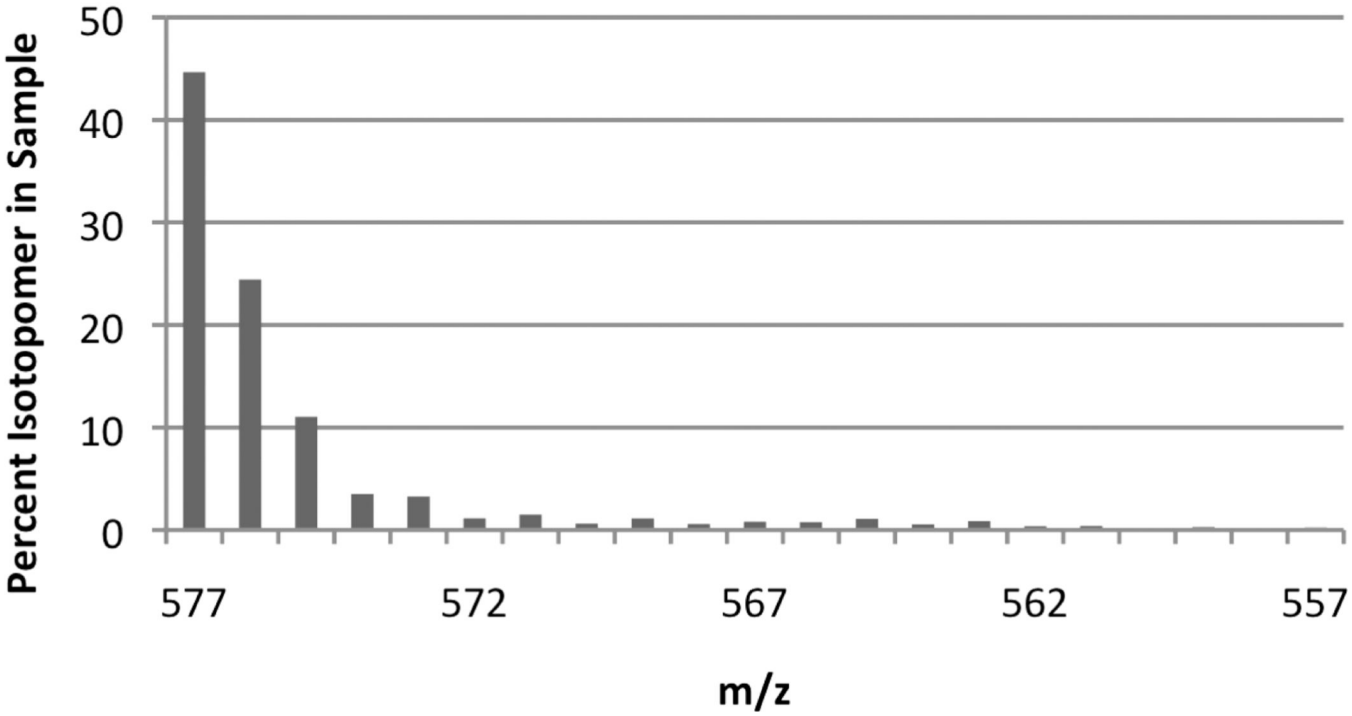

Figure 5.

Mass isotopomers present in purified ^{13}C-lycopene from the *hp-1* tomato cell culture treated with CPTA. Percent isotopomer in the sample is calculated from mass chromatogram peak signal areas obtained from four analytical injections of ^{13}C-lycopene (1000 ng each in 40 μL of diethyl ether); each injection yielded peak area signals for 6 masses selected, and sequential injections were normalized. Signals for $<m/z = 557$ were not quantifiable. In vitro labeling yields 45% uniformly labeled ($m/z = 577$) ^{13}C-lycopene and a distribution of less enriched isotopomers ($m/z = 557–576$).

Table 1

Carotenoid Concentrations from *gh* and Wild-Type-Callus Cultures (One Trial, *n* = 5 Replicates)[a]

		carotenoid concentrations (μg/g callus) ± SEM			
ghost cell line genotype	**explant tissue type**	**phytoene**	**phytofluene**	**lycopene**	**β-carotene**
wild-type	leaf	n/d	n/d	0.01 ± 0.00	0.06 ± 0.01
	flower bud	n/d	n/d	0.01 ± 0.00	0.06 ± 0.01
gh	leaf	1.41 ± 0.24	trace	0.02 ± 0.01	0.04 ± 0.00
	flower bud	5.59 ± 1.82	0.16 ± 0.04	0.03 ± 0.00	0.03 ± 0.01

[a]Carotenoid concentrations represent the average of five samples ± the standard error of the mean (SEM).

Table 2

Carotenoid Concentrations from Control *gh* and Wild-Type Cell Suspension Cultures or Treated with CPTA for the 14 Day Growth Cycle[a]

		carotenoid concentrations (μg/g cells)			
GH cell line phenotype	**herbicide treatment**	**phytoene**	**phytofluene**	**lycopene**	**β-carotene**
wild-type	CPTA	0.10	0.10	4.23	0.23
	control	n/d	trace	0.04	0.87
gh	CPTA	25.10	0.65	0.33	0.02
	control	30.87	0.75	0.19	0.17

[a]Carotenoid concentrations represent the average of two samples analyzed from one trial.

Table 3

Carotenoid Concentrations from Control *gh* and Wild-Type Cell Suspension Cultures Grown in Carotenoid Production Media for 14 Days and Treated with Norflurazon on Day 7[a]

		carotenoid concentrations (μg/g cells)			
GH cell line genotype	**herbicide treatment**	**phytoene**	**phytofluene**	**lycopene**	**β-carotene**
wild-type	norflurazon	9.53	0.65	0.05	0.41
	control	0.33	0.06	0.06	1.07
gh	norflurazon	14.08	0.18	0.11	0.15
	control	15.03	0.33	0.05	0.17

[a]Carotenoid concentrations represent the average of two samples analyzed from one trial.

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Table 4

Harvest Yields of Callus Cultures of High Lycopene Tomato Cell Lines and Available Wild-Type Cultures Grown on Agar-Solidified Carotenoid Production Medium with or without CPTA[a]

	harvest yield (g callus/3 g callus inoculum) ± SEM	
tomato cell line	**control treatment**	**CPTA treatment**
'VFNT cherry'	14.47 ± 1.79 A	13.23 ± 1.28 A
'Manapal' wild-type	6.65 ± 0.35 B	5.85 ± 0.25 B
'Manapal' *hp-2*	9.16 ± 1.42 B	8.26 ± 1.68 B
'Ailsa Craig' wild-type	7.35 ± 0.71 B	6.27 ± 0.35 B
'Ailsa Craig' *hp-1*	6.73 ± 0.23 B	5.51 ± 0.21 B
L. pimpinellifolium	8.44 ± 1.84 B	8.72 ± 0.77 B
'Suncoast' *B^og*	7.99 ± 0.35 B	7.95 ± 0.31 B

[a]The values represent the average mass yield of three trials with two reps per trial, where one rep was one container of agar-solidified medium inoculated with 3 g of callus. Statistically different yields within the treatment groups were detected by analysis of variance (α = 0.05) and are denoted by different uppercase letters.

Table 5

Carotenoid Concentrations of Control-Treated and CPTA-Treated Tomato Callus Cultures Grown on Agar-Solidified Carotenoid Production Medium[a]

	Carotenoid Concentration (μg/g cells)					
	phytoene		phytofluene		lycopene	
	control	CPTA	control	CPTA	control	CPTA
'VFNT' cherry	0.48 ± 0.22	0.89 ± 0.09	0.35 ± 0.16	0.90 ± 0.07	1.16 ± 0.56	10.90 ± 3.72
'Ailsa Craig' *hp-1*[b]	0.44 ± 0.07	2.67 ± 1.02	0.32 ± 0.05	2.12 ± 0.50	0.62 ± 0.22	40.98 ± 11.56
'Ailsa Craig' WT	0.32 ± 0.14	1.12 ± 0.15	0.23 ± 0.10	0.95 ± 0.12	0.41 ± 0.18	25.12 ± 4.28
L. pimpinellifolium	0.54 ± 0.10	0.96 ± 0.07	0.39 ± 0.07	0.70 ± 0.10	1.29 ± 0.52	15.40 ± 2.84
'Manapal' *hp-2*	0.59 ± 0.15	1.59 ± 0.34	0.43 ± 0.11	1.73 ± 0.38	1.49 ± 0.61	28.01 ± 6.41
'Manapal' WT	0.45 ± 0.20	0.85 ± 0.08	0.32 ± 0.15	0.74 ± 0.07	0.95 ± 0.51	13.31 ± 1.10
'Suncoast' B^og	0.54 ± 0.18	0.71 ± 0.01	0.39 ± 0.13	0.52 ± 0.00	0.91 ± 0.37	7.90 ± 1.56

[a] Average values of three trials with two replicate analyses per trial and SEM are presented.

[b] The CPTA-treated 'Ailsa Craig' *hp-1* cell line had a significantly greater total carotenoid (phytoene + phytofluene + lycopene) concentration than all other treatments and cell lines as detected by analysis of variance (α = 0.05).

Table 6

Carotenoid Concentrations and Harvest Yields from Tomato Cell Suspension Cultures Harvested 11 Months Post-Cell Suspension Culture Initiation[a]

	carotenoid concentration (μg/g cells)							
	phytoene		phytofluene		lycopene		harvest yield (g cells/L culture)	
	control	CPTA	control	CPTA	control	CPTA	control	CPTA
'VFNT' cherry	0.70	5.22	n/d	0.57	0.01	21.61	178	137
'Ailsa Craig' hp-1	0.50	1.69	n/d	0.20	n/d	38.09	270	199
'Ailsa Craig' WT	0.01	0.62	n/d	0.09	n/d	39.52	192	138
'Manapal' hp-2	0.01	0.73	n/d	0.09	n/d	27.55	131	84
'Manapal' WT	0.01	0.17	n/d	0.03	n/d	10.63	168	147

[a]Cultures were treated with or without CPTA and harvested after one 14 day growth period. Each value represents the average of two samples. Samples with either no signal for a carotenoid or a signal below accurate quantification are denoted as not detectable (n/d).