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Introducing Selective Agrochemical Manipulation of Gibberellin Metabolism into a Cereal Crop

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Abstract

Use of growth retardants enables post-planting optimization of vegetative growth, which is particularly important with the on-going climate change. Mepiquat chloride is an economical and safe retardant widely applied in cotton, but is not uniformly effective. Here identification of its molecular target as the ent-copalyl diphosphate synthase that initiates gibberellin biosynthesis enabled introduction of selective agrochemical inhibition, leaving intact more specialized metabolism important for resistance to biotic and abiotic stresses.

The gibberellin A (GA) phytohormones play a key role in promoting shoot elongation and vegetative growth¹. Accordingly, GA metabolism is a critical agrochemical target as many

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Author contributions

J.Z. made all constructs, carried out all experiments in *Arabidopsis* and *in vitro* biochemical assays. Y.Z. carried out maize growth inhibition experiments. J.X. assisted with transgenic maize screening. H.Y. and R.Z. carried out the experiments with cotton. M.Z. guided the transgenic plant experiments. Y.C. and Q.W. measured maize kauralexin levels. D.Z. and P.Y. attempted expression of *GhCPS* in insect cells. J.Z., Y.Z. and R.J.P. were primarily responsible for writing the manuscript. R.J.P. and Z.L. supervised the research and aided in data analysis. Z.L. conceived of the project.

Competing financial interests

The authors declare a financial interest as Z.L. has filed a patent related to the results reported here.

Additional Information

Supplementary Figures are available in the online version of the paper.

agriculturally relevant plant-growth retardants, often used as anti-lodging agents in cereal crop plants, have been shown to act by inhibiting GA biosynthesis².

GAs are diterpenoids originating from (*E,E,E*)-geranygeranyl diphosphate (GGPP), which is initially cyclized to *ent*-copalyl diphosphate (*ent*-CPP) by an *ent*-CPP synthase (CPS), with further cyclization catalyzed by *ent*-kaurene synthase (KS), followed by extensive elaboration catalyzed by cytochrome P450 (CYP) mono-oxygenases and, subsequently, 2-oxoglutarate dependent di-oxygenases (2ODDs)³. Both CYPs and 2ODDs form large enzymatic families in plants that are employed by many other metabolic processes as well^{4,5}. Thus, while GA biosynthesis can be blocked by general inhibitors such as structural mimics of 2-oxoglutarate and nitrogenous heterocycles, which target 2ODDs and CYPs, respectively, these also retard other essential metabolism².

Beyond the oxygenases, the CPS and KS from GA biosynthesis have repeatedly undergone gene duplication and neo-functionalization to give rise to more specialized (di)terpenoid metabolism that typically plays important roles in plant defense⁶. Although structurally and mechanistically distinct, these cyclases both catalyze carbocationic cascade reactions⁷. Hence, it has been proposed that their inhibition by positively charged compounds such as the quaternary ammonium-containing mepiquat chloride (1,1-dimethyl-piperidinium chloride, DPC), chlormequat chloride and Amo 1618, is due to electronic analogy of these compounds to the relevant high-energy intermediates², although there is little other structural similarity. However, at least the latter two of these agriculturally-utilized plant growth inhibitors largely target CPS rather than KS⁸.

The CPS from GA metabolism exemplifies the enzymatic family of class II diterpene cyclases, which initiate biosynthesis of the large (~7,000 known) labdane-related diterpenoid natural product super-family⁹. These compounds are particularly important in cereal plant defense¹⁰. Accordingly, the use of general inhibitors of class II diterpene cyclases might be expected to block not only plant growth (i.e., via the targeted GA biosynthesis), but defense as well (i.e., via inadvertent interference with more specialized labdane-related diterpenoid metabolism), particularly in this critical crop plant family. Thus, while offering the advantage of post-planting control of vegetative growth, which will be of increasing agronomic value given the variability in weather associated with climate change, such agrochemical manipulation of GA biosynthesis might also impose adverse effects due to inhibition of important more-specialized metabolic processes as well. Intriguingly, there is evidence of selectivity for at least some GA inhibitors. In particular, while highly effective in cotton (*Gossypium hirsutum*) where it is widely used, DPC is much less potent in many other plants – e.g., rice (*Oryza sativa*) and maize (*Zea mays*)¹¹.

Here the selective nature of DPC inhibition was investigated. Based on the working hypothesis that DPC targets CPS, the recently identified cotton *GhCPS*¹² was expected to be sensitive to inhibition. Given the reported insensitivity of maize, which was verified here (Supplementary Fig. S1), and the early identification of the *ZmCPS1* (An1) primarily associated with GA metabolism in maize¹³, this was expected to be insensitive. Their susceptibility was examined in a transgenic approach using the model plant *A. thaliana* for which there are known *AtCPS* (GA1) mutants, with both severely (*gal-1*) and moderately

(*gal-5*) dwarfed lines available¹⁴. Given that the parental/wild-type (WT) *A. thaliana* var. *Landsberg erecta* (Arabidopsis) is moderately susceptible to growth inhibition by DPC (and consistent with the expected effect this can be reversed by application of bioactive GA; Supplementary Fig. S2), in addition to *GhCPS* and *ZmCPS1*, *AtCPS* also was expressed in both *gal-1* and *gal-5* Arabidopsis. As expected, all three CPSs were able to complement the dwarf phenotype of both *gal* mutants (Supplementary Fig. S3). Consistent with its ability to genetically complement *gal*, *GhCPS* is correctly localized to plastids/chloroplasts (Supplementary Fig. S4). Strikingly, these transgenic lines exhibited very different responses to DPC. While the *gal* lines expressing *AtCPS* exhibited moderate susceptibility to growth retardation similar to WT Arabidopsis (Extended Data Fig. E1), the *ZmCPS1* complemented lines are almost completely resistant and the *GhCPS* complemented lines were significantly more sensitive (Fig. 1). Analogous expression of these CPSs in WT Arabidopsis confirmed the dominant role of CPS susceptibility to the effect of DPC on plant growth (Extended Data Fig. E2). Thus, DPC not only seems to target CPS, but also do so in a selective manner.

To further investigate the utility of *GhCPS* for introduction of selective growth retardation by DPC, a similar transgenic approach was employed in maize. To generate the appropriate genetic background *ZmCPS1/An1* was targeted for mutagenesis via the CRISPR/Cas9 method. As previously reported¹³, homozygous *zmcp1/an1* knock-out mutants exhibit severe defects in seed development, and the ear sheath essentially blocks application of bioactive GA₃, complicating propagation. Thus, the work reported here was carried out with diallelic *zmcp1/an1* mutant plants, with one allele carrying a single base insertion and the other an in-frame three-base deletion, as the latter apparently is not disruptive (i.e., plants homozygous for this single amino acid deletion exhibited normal growth and development), that do exhibit impaired growth (Supplementary Fig. S5). Diallelic embryos were then transformed with a *GhCPS* expression construct, and the resulting expression levels measured (Extended Data Fig. E3). In contrast to WT maize, these transgenic plants do respond to DPC (Extended Data Fig. E4), particularly those that remain diallelic (Fig. 2), which also exhibited higher *GhCPS* expression levels. Although the effect is limited, this presumably reflects the fact that *an1* mutants only exhibit a semi-dwarf phenotype¹³, which is due to the presence of another maize CPS (*An2/ZmCPS2*) that produces *ent*-CPP¹⁵ and must partially complement the loss of *An1/ZmCPS1* in *an1* plants as the other maize CPSs exhibit different activity¹⁶. The limited effectivity of DPC in these transgenic maize plants suggests that *ZmCPS2* also is insensitive. Importantly, DPC application did not reduce grain numbers or weight (Extended Data Table E1), demonstrating that DPC sensitivity can be introduced into maize without impacting yield.

Unlike Arabidopsis, maize exhibits more specialized labdane-related diterpenoid metabolism, which plays important roles in response to both biotic and abiotic stresses. In particular, maize utilizes *An2/ZmCPS2* for production of kauralexins, which play an important role in not only defense against fungal pathogens and rootworm herbivory, but also in drought resistance¹⁷. Given the suggestion that *ZmCPS2* is resistant, it seemed likely that kauralexin production should be unaffected by DPC, which was verified here (Extended Data Fig. E5).

To further characterize the inhibition of CPS by DPC mechanistic studies with the highly susceptible *GhCPS* were attempted. Unfortunately, recombinant *GhCPS* was found to be unstable *in vitro*. Nevertheless, when *GhCPS* was expressed in *E. coli* also engineered to make its substrate GGPP¹⁸, the production of *ent*-copalol, derived from dephosphorylation of *ent*-CPP by endogenous phosphatases, was observed (Supplementary Fig. S6). Whole-cell inhibition studies with these cultures indicated that *GhCPS* activity was effectively blocked by DPC (Extended Data Fig. E6).

Fortuitously, *AtCPS* has long since been identified¹⁹, shown to be amenable to enzymatic studies *in vitro*²⁰, and seems to be at least somewhat susceptible to inhibition by DPC (Supplementary Fig. S2). This was further demonstrated by comparison of the ability of DPC to inhibit *AtCPS* versus *ZmCPS1* or *ZmCPS2* *in vitro* (Extended Data Fig. E7), revealing that *AtCPS* is ~100-fold more susceptible than the maize CPSs (i.e., IC₅₀ of 0.03 versus 2 or 4 mM, respectively). For direct comparison these CPSs also were incorporated into the engineered *E. coli* system for whole-cell DPC inhibition studies, and DPC found to most effectively inhibit *GhCPS* in this system as well (Extended Data Fig. E8).

To provide mechanistic insight into DPC inhibition *AtCPS* was subjected to additional *in vitro* analysis (Extended Data Fig. E9). While complicated by the known substrate inhibition²⁰, DPC does not appear to be a competitive inhibitor, as it affects both productive and non-productive (inhibitory) substrate binding (K_M and K_{si} , respectively), along with catalytic rate (Table 1). Hence, DPC does not mimic carbocation intermediates of the catalyzed reaction, nor necessarily bind in the active site at all. Indeed, DPC may be an uncompetitive inhibitor as it seems to decrease K_M , although increase K_{si} , suggesting it may selectively bind the enzyme-substrate complex and act through an allosteric mechanism. Accordingly, DPC presumably binds elsewhere and indirectly inhibits CPS activity by altering protein structural conformation. Given the differences between the readily inhibited *GhCPS* and resistant *ZmCPSs* (~51% amino acid sequence identity), as well as the further confounding intermediate susceptibility of *AtCPS*, it has not yet been possible to identify the DPC binding site, let alone the structure-function relationships that underlie susceptibility.

Although it has been previously suggested that the more general inhibition exerted by non-specific plant growth retardants is an acceptable side-effect², this also blocks metabolic responses that may be important for adaptation to adverse conditions. Accordingly, in order to most effectively use such agrochemicals to ameliorate the effects of adverse weather patterns, such as those expected to result from climate change (e.g., to pause growth during unexpected periods of drought or flooding), the introduction of susceptibility to selective inhibitors such as DPC (as shown here) seems a fruitful avenue of investigation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Landsberg erecta* accession of *Arabidopsis thaliana* was used unless stated otherwise. The mutants *gal-1* (CS58) and *gal-5* (CS3106) were ordered from the Arabidopsis Biological Resource Center (www.arabidopsis.org).

Seedlings were grown on Murashige and Skoog (MS) medium (Phyto Technology Laboratories) containing 3% (w/v) Suc and 0.8% (w/v) agar (and, for the non-complimented *gal* mutants, treated with GA₃ – sprayed with a 10⁻⁶ M solution) for 5 to 7 d, then transplanted into pots (7 × 7 × 7 cm) containing soil (PINDSTRUP SPHAGNUM MOSS PEAT). The potted plants were kept at 22 °C with illumination at 120 μmol m⁻²s⁻¹ for a 16 h light and 8 h dark cycle. The relative humidity was ~ 70% (±5%). Non-complimented *gal* mutant seedlings were treated with GA₃ every two days (foliar spray with 10⁻⁶ M solution).

Maize B73–329 was used for all the studies described here. Seeds were grown in square plastic pots (20 × 20 × 30 cm in height) containing a mixture of vermiculite and commercial garden soil (1:1; v/v) and grown in a greenhouse cycling between 14 h light at 25 °C and 10 h dark at 18 °C.

Cotton (*Gossypium hirsutum* L., *Xinkang 4*) was grown in round pots (13 cm diameter and 13 cm in height) containing a mixture of vermiculite and commercial garden soil (1:1; v/v) in a glass greenhouse cycling between 14 h light at 28 °C and at 10h dark at 20 °C.

Construction of transgenic Arabidopsis, Subcellular Localization and Treatment

The full-length cDNA of *GhCPS*, *ZmCPS1* and *AtCPS* were individually fused upstream of GFP under the control of the super promoter in the pCambia1300 vector, as previously described²¹. The forward primer used for *GhCPS* introduced an XbaI restriction site (5'-CTAGTCTAGAATGTTTCCCATTCCTTCCT-3'), while the reverse introduced a KpnI restriction site (5'-CGGGGTACCGCGTACTTTCTCAAAG-3'). The forward primer used for *ZmCPS1* introduced a SpeI restriction site (5'-CGGACTAGTATGAAGCTCCTCTCGCCGGC-3'), while the reverse introduced a KpnI restriction site (5'-CGGGGTACCTTAAACAGGCTCAAAGATAACTCT-3'). The forward primer used for *AtCPS* introduced a SpeI restriction site (5'-CGGACTAGTATGTCTTTCAGTATCATGTTC-3'), while the reverse introduced a KpnI restriction site (5'-CGGGGTACCGACTTTTTGAAACAAGACT –3').

Each of these vectors was separately transformed into *Agrobacterium tumefaciens* strain GV3101, and the resulting strain then used to transform Arabidopsis plants (WT, *gal-1* and *gal-5*) by the floral dip method²². Transformed seedlings were selected on MS plates containing 30 mg/L hygromycin. The described studies were carried out with seedlings of the T2 generation of hygromycin-resistant and single copy containing plants.

GhCPS Subcellular Localization

To visualize *GhCPS*-GFP sub-cellular localization, the roots of seven-day-old transgenic Arabidopsis plants were transferred onto glass slides, covered with slips, and observed under a confocal laser microscope (FV1000, Olympus, Japan) with an excitation of 488 nm and an emission of 500–550 nm. The observed punctate pattern of *GhCPS*-GFP contrasts with the more uniform distribution of GFP and was interpreted as reflecting import into plastids.

Super1300:*GhCPS*-GFP vector was transformed into *A. tumefaciens* strain EHA105 and infiltrated into leaves of *N. benthamiana*. After 3 days incubation at 25 °C, the tobacco leaves were used for fluorescence signal observation. Green and red fluorescence were

observed under a confocal laser microscope (FV1000, Olympus, Japan). GFP was excited with a 488 nm laser. The emission spectra were collected at 500–550 nm for GFP. To detect auto-fluorescence of chlorophyll, samples were examined with a long-pass 630 nm filter set, as previously described²³. The overlapping red and green fluorescence demonstrates localization of *GhCPS*-GFP into chloroplasts.

DPC treatment

Cotton was treated with DPC (foliar spray with solutions of 0, 30, 50, 100, 300, 500, 1000 mg/L) at the two-leaf stage. Plant height was measured 15 days after this treatment. Each treatment set contained 15 replicates.

For *Arabidopsis*, seeds were germinated on MS plates for 10 days and then transferred to pots with soil. DPC (CAS 24307–26-4, Sigma-Aldrich, USA) treatment (foliar spray with solutions of 0, 30, 50, 100, 300, 500, 1000 mg/L) was started upon bolting, with selection of plants containing equal numbers of leaves. To demonstrate that GA can reverse DPC growth inhibition, WT plants were treated with 500 mg/L DPC and then GA₃ (foliar spray with 10^{−6} M solution every two days). Plant height was then measured 15 days after this treatment. Each treatment set consisted of at least 7 replicates.

For analysis of effect on maize growth and development, plants were treated with DPC (foliar spray) at the 9-leaf growth stage (specifically when the 9th internode was 1 cm in length). Five concentrations of DPC were used for the sensitivity assay (0, 50, 100, 500, 1000 mg/L), with 500 mg/L DPC used for comparison among transgenic maize, *zmcp1* mutant and WT (B73–329). Plant and ear heights were measured at the silking stage, ~22–25 days after treatment. Following maturation, each ear was harvested separately and naturally air-dried under room condition. Then the grains were threshed from each ear by hand. The number of grains per ear were counted and the total grain weight per ear also measured. The 100-grain weight was calculated by the rate of total grain weight to number of grains per ear. Each treatment set consisted of 10 replicates.

For analysis of effect on maize kauralexin production, seeds were sterilized, germinated and seedlings grown hydroponically, as previously described²⁴. When the third leaf was fully expanded, the seedlings were individually transferred into a glass bottle (height 15 cm × 5 cm diameter) containing 150 mL full strength Hoagland's nutrient solution, and treated with water or 1000 mg/L DPC (foliar spray). A week later, leaves, roots and roots exudates were harvested separately. The leaves and roots were separately ground to fine powder in liquid N₂ and extracted twice with hexanes. This organic extract was concentrated by rotary evaporation and methylated with CH₂N₂ for GC-MS analysis. For root exudate analysis, the spent media was loaded onto 0.1 g Bond Elut C18 spin-cartridges (Thermo, USA), which were then eluted with 10 ml of 100% ethyl acetate. This eluate was dried under N₂ and the residue dissolved in hexanes, methylated as above, then analyzed by GC-MS. Kauralexin content was quantified by peak size (extracted ion abundance) based on a standard curve generated with an authentic standard for kauralexin B1. Each treatment set consisted of 5 replicates.

One-way analyses of variance (ANOVA) procedure was conducted with SAS (V8, SAS Institute Inc., Cary, NC, USA). Multiple comparisons among different lines and treatments were corrected based on Fisher's protected least significant difference (LSD). Student's unpaired t-tests were used for pairwise comparisons. P values < 0.05 and/or 0.01 were considered significant.

Construction of transgenic maize

The *CRISPR-Cas9* (clustered regularly interspaced short palindromic repeat-CRISPR-associated protein 9) knockout lines were created by the Research Center for Functional Genomics and Crop Breeding of China Agricultural University according to previous methods^{25,26}. Briefly, a pCambia-derived CRISPR/Cas9 binary vector with two *gRNAs*: GAAATTGCGAAATGGCCAG (target1) and AGCTGAAGCGGATCCCAAG (target2) expression cassettes targeting two adjacent sites of *ZmCPS1* was generated and then transformed into *A. tumefaciens* strain EHA105. The resulting recombinant strain was then transformed into maize by infection of immature embryos from the B73-329 inbred line. In order to identify *CRISPR-Cas9* induced mutations, the *gRNA*-targeted sites were sequenced with the following primers: 5'-ATCAACCGTACTGCTGCAAACAG-3' and 5'-CGCTAGCCTCCACATGTTCT-3' for target 1, and 5'-AAGACCTTGAGGATGAGCACCAG-3' and 5'-AAGTAGCGGGAGATCCCGAGT-3' for target 2. Given the difficulties with homozygous knock-out mutants of *zmcp1/an1*, diallelic (single base insertion and three-base deletion) plants were used for propagation. The *A. tumefaciens* strain EHA105 containing the Super1300-*GhCPS* vector was used to infect immature embryos from the seeds of such a diallelic *zmcp1/an1* plant (T1 generation). Resulting transgenic (diallelic) *zmcp1/an1 + GhCPS* plants were selected by sequencing and PCR (of *GhCPS*), with the next generation of plants used in the experiments described here (with sequencing of the *zmcp1* loci to identify genotype). *GhCPS* expression levels were analyzed by real-time quantitative PCR, carried out as previously described²⁴, using actin as a control with the following primers: ZmActin-F: GTTCCCTGGGATTGCCGAT; ZmActin-R: CTGCTGAAAAGTGCTGAG; GhCPS-qRT-F: TCCTCCCTTACCCTTTGCAG; GhCPS-qRT-R: GCATATTCTTGAGTGCGGGG. Control plants were from the corresponding (T3) generation and also sequenced to identify their *zmcp1* genotype.

Characterization of GhCPS

For recombinant expression *GhCPS* was truncated (removing the first 94 amino acids) to create a pseudo-mature construct, which was sub-cloned into the Gateway vector system via PCR amplification and directional topoisomerase-mediated insertion into pENTR/SD/D-TOPO and verified by complete sequencing. An analogous truncated pseudo-mature *AtCPS* in pENTR has been previously described²⁰, as have the similar *ZmCPS1* and *ZmCPS2* constructs¹⁵. Each of these CPSs were individually transferred via directional recombination to the previously described T7 promoter-based expression vector pGG-DEST¹⁸. A codon-optimized *GhCPS* gene was also synthesized and incorporated into the Bac-to-Bac Baculovirus expression system (Thermo-Fisher) as previously described²⁷, but no activity was observed with the resulting recombinant protein upon purification, or even with cell-free extracts.

For whole-cell inhibition studies a previously described modular metabolic engineering system was used¹⁸. All metabolic engineering was carried out using the *E. coli* OverExpress C41 strain (Lucigen), and included pIRS to increase flux to isoprenoid metabolism as previously described²⁸, with pDEST17-AtKS included in the experiment verifying the stereochemical product outcome of *GhCPS*. Recombinant cultures were grown in 50 mL TB medium (pH 7.0), with the appropriate antibiotics, in 250 mL Erlenmeyer flasks. These cultures were firstly grown at 37 °C to mid-log phase ($OD_{600} = 0.6\text{--}0.8$), then the temperature dropped to 16 °C for 0.5 – 1 h prior to induction with 1 mM isopropylthiogalactoside (IPTG) and supplementation with 40 mM pyruvate and 1 mM $MgCl_2$. To examine the effect of DPC this was added to final concentrations of 0, 30, 50, 100, 300, 500 or 1000 mg/L, with 3 replicates for each. The induced cultures were grown for an additional 3 days before extraction with an equal volume of hexanes. The extracts were dried under N_2 and then resuspended in 1 mL fresh hexanes for analysis.

For characterization and quantification of the products, gas chromatography with mass spectra detection (GC-MS) was carried out with a 3900 GC with Saturn 2100T ion trap MS (Varian) equipped with HP-5 MS column (Agilent). Samples (1 μ L) were injected in splitless mode by an 8400 autosampler with the injection port at 250 °C, the column oven temperature initially started at 50 °C, which was maintained for 3 min, and then increased at a rate of 15 °C/min to 300 °C, where it was held for another 3 min. Mass spectra were recorded by mass-to-charge ratio (m/z) values in a range from 90 to 650, starting from 13 min after sample injection until the end of the run. The production of *ent*-copalol, derived from dephosphorylation of *ent*-CPP by endogenous phosphatases, and (where relevant) *ent*-kaurene, was verified by comparison of retention time and mass spectra to that of authentic standards.

Inhibition of AtCPS and ZmCPSs

Expression vectors (pDEST-17 based) for truncated pseudo-mature *AtCPS*, as well as *ZmCPS1* and *ZmCPS2*, also have been previously described^{15,20}. These were transformed into the C41 OverExpress strain of *E. coli* (Lucigen) for production and isolation of each CPS much as previously described²⁹. Briefly, the recombinant *E. coli* were grown in liquid NZY media to $OD_{600} = 0.8$ at 37 °C, shifted to 16 °C for prior 0.5–1 h to induction with 1 mM IPTG, then grown overnight. The cells were harvested and lysed using a homogenizer. The proteins were purified with 2 mL nickel-nitrilotriacetic acid superflow resin column using a BioLogic LP chromatography system (BioRad). All steps of the purification were carried out at 4 °C and resulting purity of the CPSs examined by SDS-PAGE analysis (each ~90% pure). The proteins were then dialyzed in 50 mM MOPSO (pH 6.8) and 2 mM DTT overnight at 4 °C, then aliquots were flash frozen in liquid N_2 and stored at –80 °C.

All enzymes assays were carried out at 30 °C in 1 mL of assay buffer (50 mM HEPES (pH 7.75), 100 mM KCl, 10% glycerol (v/v) and 0.01 mM $MgCl_2$) with appropriate CPS, as well as GGPP and DPC. For time course assays, each assay was incubated with 5 μ M GGPP and different concentrations of CPS (0.01, 0.02, 0.05, 0.1 μ M) for 15, 30, 60 or 120 sec. For kinetics assays, each assay was incubated with different concentrations of GGPP (0.5, 1, 2, 3, 5, 7.5, 10, 12.5, 15, 20 or 30 μ M) and appropriate CPS and time. For IC_{50} assays, each

assay was incubated with CPS and GGPP for the appropriate time and with various concentrations of DPC (0, 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} or 10^0 M). Reactions were terminated by adding 100 μ L of 20 mM NEM in 500 mM glycine (pH 11), then incubating for 5 min at 75 °C. Excess NEM was deactivated by adding 20 μ L 1 M DTT and incubating for 15 min at room temperature, followed by neutralization with 60 μ L 1M HCl. Dephosphorylation to enable analysis by GC was carried out by the addition of 133 μ L of $10 \times$ CIAP buffer (Promega) and 15 U calf intestinal alkaline phosphatase, with incubation at 37 °C overnight. The resulting alcohols (geranylgeraniol and *ent*-copalol) were extracted with 1 mL hexanes thrice, with the extracts from each assay pooled, dried under N_2 and resuspended in 50 μ L hexanes for GC analysis. After initial product verification by GC-MS, catalytic turnover was quantified by GC-FID (flame ionization detection). Kinetic parameters were calculated from fitting the observed data to the substrate inhibition equation (Kaleida Graph 4.0, Synergy Software; Reading, PA).

Extended Data

Extended Data Figure E1.

DPC can retard growth of *ga1* mutants of Arabidopsis recombinantly expressing *AtCPS*. A) *ga1-1* + *AtCPS* and B) *ga1-5* + *AtCPS* plants treated with the indicated concentrations of DPC (vertical red bars = 2 cm). C) Histogram of plant heights (n=7; means $\pm$ s.d., while letters (a – f) indicate significant differences – i.e. $P < 0.05$ for two-sided Fisher's LSD – dots indicate values measured for individual plants).

Extended Data Figure E2.

DPC can retard growth of WT Arabidopsis recombinantly expressing *AtCPS* or *GhCPS*, but not *ZmCPS1*. Growth curves are from A) WT; B) *Super 1300-GFP*+WT; C) *Super 1300-GFP-GhCPS*+WT; D) *Super 1300-GFP-ZmCPS1*+WT; E) *Super 1300-GFP-AtCPS*+WT plants to which DPC (500 mg/L) has been applied (n=7; means $\pm$ s.d.). Plant height was measured every two days after DPC treatment.

Extended Data Figure E3.

Relative expression level of *GhCPS* in transgenic maize lines used here (n=3; means $\pm$ s.d., dots indicate values measured for technical replicates).

Extended Data Figure E4.

Effect of DPC on homozygous *zmcp1/an1* transgenic maize, also expressing *GhCPS*. Pictured are A) *GhCPS* in *zmcp1* MU (444); B) *GhCPS* in *zmcp1* MU (673) plants to which DPC (500 mg/L) has been applied, as well as C) a histogram showing plant (Left) and ear (Right) height ($n=10$; means $\pm$ s.d., with significant differences (using two-sided Student's t-test) indicated by shown P values, dots indicate values measured for individual plants).

Extended Data Figure E5.

DPC does not affect kauralexin (phytoalexin) production in maize (n=5; means $\pm$ s.d., dots indicate values measured for individual plants).

Extended Data Figure E6.

DPC can inhibit production of *ent*-CPP by *GhCPS* in *E. coli* also engineered to produce GGPP (n=3; means ± s.d., with significant differences – i.e. $P < 0.05$ for two-sided Fisher's LSD – dots indicate values measured for individual cultures).

Extended Data Figure E7.

DPC is a selective CPS inhibitor. Effect of increasing DPC concentration on the activity of A) *AtCPS*; B) *ZmCPS1*; or C) *ZmCPS2*. Average from three independent measurements, error bars represent standard deviation. Lines indicate fit to $y = A_1 + (A_2 - A_1) / (1 + 10^{-(\text{LOG}x_0 - x) * p})$.

Extended Data Figure E8.

DPC has a highly variable effect on production of *ent*-CPP by distinct CPSs in *E. coli* also engineered to produce GGPP (n=3; means $\pm$ s.d., with significant differences – i.e. $P < 0.05$ for two-sided Fisher's LSD – dots indicate values measured for individual cultures).

Extended Data Figure E9.

Effect of DPC on kinetic assays with *AtCPS*. Plot of observed reaction rate with varying concentrations of GGPP in the presence of A) 0 B) 10 or C) 100 μM DPC. Average from three independent measurements, error bars represent standard deviation. Line depicts fit to the substrate inhibition formula as previously described²⁰.

Extended Data Table E1.

Grain yield component of different lines following DPC treatment

Line	Treatment	Grain number per ear	100 grain weight (g)	Yield per plant (g)
WT	CK	130 b	25.57 a	33.31 b
	DPC	131 b	25.34 a	33.10 b
443	CK	117 b	23.08 b	27.12 c
	DPC	120 b	23.66 ab	28.60 c
589	CK	170 a	22.15 b	37.63 a
	DPC	172 a	22.11 b	37.57 a

Values are the means ($n=7$). Different letters in the same column indicate significant differences between the lines and/or treatments ($P < 0.05$; using two-sided Fisher's LSD).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

DPC targets CPS. Effect of DPC on transgenic Arabidopsis (*gal* mutants) expressing either *ZmCPS1* or *GhCPS*. Pictured are A) *gal-1* + *ZmCPS1*; B) *gal-5* + *ZmCPS1*; C) *gal-1* + *GhCPS*; D) *gal-5* + *GhCPS* plants to which DPC has been applied at the indicated concentrations (vertical red bars = 2 cm), as well as E) histograms of plant heights (n=7; means $\pm$ s.d., while letters (a – f) indicate significant differences – i.e. $P < 0.05$ for two-sided Fisher's LSD – dots indicate values measured for individual plants).

Figure 2.

Introducing DPC sensitivity into maize. Effect of DPC on transgenic maize, specifically *zmcp1/an1* mutants also expressing *GhCPS*. Pictured are A) *GhCPS* in *zmcp1* MU (443); B) *GhCPS* in *zmcp1* MU (589) plants to which DPC (500mg/L) has been applied, as well as C) a histogram showing plant (Left) and ear (Right) heights (n=10; means $\pm$ s.d., with significant differences (using two-sided Student's t-test) indicated by shown P values, dots indicate values measured for individual plants).

Table 1.Effect of DPC on *AtCPS* kinetic constants¹

	0	10 μM DPC	100 μM DPC
k_{cat} (s^{-1})	0.3 ± 0.2	0.22 ± 0.06	0.09 ± 0.02
K_M (μM)	7 ± 5	4 ± 2	3 ± 1
K_{si} (μM)	5 ± 4	12 ± 6	60 ± 40
R^2	0.92	0.90	0.95

¹Kinetic parameters calculated from fitting the observed data to the substrate inhibition equation (KaleidaGraph 4.0; Synergy). Values indicate the average $\pm$ standard error from the curve fit. The experiment was independently repeated twice with similar results.