Engineered gray mold resistance, antioxidant capacity, and pigmentation in betalain-producing crops and ornamentals

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Betalains are tyrosine-derived red-violet and yellow plant pigments known for their antioxidant activity, health-promoting properties, and wide use as food colorants and dietary supplements. By coexpressing three genes of the recently elucidated betalain biosynthetic pathway, we demonstrate the heterologous production of these pigments in a variety of plants, including three major food crops: tomato, potato, and eggplant, and the economically important ornamental petunia. Combinatorial expression of betalain-related genes also allowed the engineering of tobacco plants and cell cultures to produce a palette of unique colors. Furthermore, betalain-producing tobacco plants exhibited significantly increased resistance toward gray mold (*Botrytis cinerea*), a pathogen responsible for major losses in agricultural produce. Heterologous production of betalains is thus anticipated to enable biofortification of essential foods, development of new ornamental varieties, and innovative sources for commercial betalain production, as well as utilization of these pigments in crop protection.

betalains | biofortification | secondary metabolism | metabolic engineering | plant biotechnology

Betalains are water-soluble, nitrogen-containing plant pigments that are synthesized from tyrosine. The betalain class contains a wide array of compounds, which are generally classified into two groups: the red betacyanins and the yellow betaxanthins (1). Betalains have attracted both scientific and economic interest (2–4). They have long been in use as food colorants, with pokeberry juice being used as early as the 19th century to enhance the color of red wine (5). Their stability in a wide pH range has also made them a pigment of choice for the food industry today, in which they are widely used as natural dyes for dairy, confectionery, and meat products (4). Their noted antioxidant properties (6, 7) have led to commercialization of a variety of betalain-based products in the dietary supplements industry and have prompted extensive studies into their potential health-promoting properties, including anticancer, hypolipidemic, antiinflammatory, hepatoprotective, and antidiabetic activities (8–13).

In the plant kingdom, betalains are limited to a single order, Caryophyllales, and found in very few edible plants, with red beet being the only major source for betalain extraction in commercial use today (14). Their beneficial biological activities, together with their limited availability in nature and particularly in the human diet, have sparked research into characterization of the betalain biosynthetic process, as well as the attempted production of betalains in naturally nonproducing organisms. However, progress in heterologous betalain engineering was hindered primarily due to the lack of knowledge of the gene(s) involved in the first step of the pathway, namely, 3-hydroxylation of tyrosine to form L-DOPA (15). Attempts for biotechnological betalain production were therefore focused on development of *Beta vulgaris* cell or hairy root culture (16). Likewise, the wide range of colors that can be produced by betalains makes them an excellent target for development of new ornamental varieties. However, as genetic engineering of

betalains has been obstructed due to the lack of a fully decoded biosynthetic pathway, genetic engineering of flower color has thus far focused on modification of pathways generating flavonoid/anthocyanin or carotenoid pigments (17).

In a recent study we reported on two cytochrome P450-type enzymes, *CYP76AD1* and *CYP76AD6*, that act redundantly to catalyze the first step of betalain biosynthesis in red beet, namely, the 3-hydroxylation of tyrosine to form L-DOPA (18). Elucidation of the last unknown step in the core pathway demonstrated the ability for stable, heterologous betalain production in tobacco (*Nicotiana tabacum*). More specifically, expression of three genes from the pathway, namely, the cytochrome P450 *CYP76AD1*, *BvDODA1* dioxygenase, and the *cDOPA5GT* glycosyltransferase, in a single binary vector (pX11), resulted in entirely red, betalain-accumulating tobacco plants (18).

Here we conducted metabolic engineering for betalain production in three important food crops: tomato, potato, and eggplant, as well as in the ornamental plant species *Petunia × hybrida*. Additionally, tobacco plants with various flower colors were generated via expression of different combinations of betalain-related genes, resulting in accumulation of betalains in different betacyanin/betaxanthin ratios. Stable betacyanin and betaxanthin production without substrate feeding was also attained in tobacco (BY-2) cell-suspension culture. Finally, we report significantly increased resistance in betalain-producing transgenic tobacco plants against leaf infection by gray mold fungus (*Botrytis cinerea*), a necrotrophic plant pathogen that infects more than 200 plant species and is responsible for annual

Significance

In plants, three major classes of pigments are generally responsible for colors seen in fruits and flowers: anthocyanins, carotenoids, and betalains. Betalains are red-violet and yellow plant pigments that have been reported to possess strong antioxidant and health-promoting properties, including anticancer, antiinflammatory, and antidiabetic activity. Here, heterologous betalain production was achieved for the first time in three major food crops: tomato, potato, and eggplant. Remarkably, betalain production in tobacco resulted in significantly enhanced resistance toward gray mold (*Botrytis cinerea*), a plant pathogen responsible for major crop losses. Considering the significant characteristics of these molecules, heterologous betalain production now offers exciting opportunities for creating new value for consumers, producers, and suppliers of food crops and ornamental plants.

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Fig. 3. Constitutive, fruit-specific, or anthocyanin coproduction of betalains in tomato. (A) Transformation of the pX11 vector resulted in formation of violet-red pigmented tomato plants (pX11-Mt). Unripe pX11-Mt fruit are shown (*Left*). pX11-Mt was crossed with large-fruited, commercial tomato (cv. M82). Ripe fruit are shown (*Top Right*). Introduction of the pX11(E8) vector into a cv. M82 tomato background resulted in pigmentation restricted to ripe fruit (*Bottom Right*). (B) Antioxidant capacity in ripe wild-type and pX11-Mt fruit analyzed by the TEAC assay. Average values of three biological replicates per genotype are shown, with error bars representing SEM. FW, fresh weight. ****P* value < 0.001 (*t* test). (C) Crossing pX11 with the Del/Ros1 lines. (*Top*) Unripe fruit. (*Bottom*) Ripe fruit. (D) MALDI-MSI of ripe fruit of the four genotypes presented in C. Betanin (*m/z* 551.1513), petunidin (*m/z* 317.0661), lycopene (*m/z* 537.4460), chlorophyll a (*m/z* 892.5348), and naringenin chalcone (*m/z* 273.0758). (Scale bar, 2 mm.)

Fruit-Specific Betalain Production in Commercial Tomato. To examine the possibility of betalain production in a commercial tomato variety, the pX11-Mt line was subsequently crossed with the processing-tomato cv. M82. pX11-Mt × M82 plants exhibited a similar visual phenotype to the one observed in pX11-Mt, in which fruit and vegetative tissues are pigmented, and fruit color changes from pink to dark red during ripening (Fig. 3A). Constitutive production of tyrosine-derived compounds such as betalains could pose a significant metabolic constraint on engineered plants in terms of precursor availability. We therefore generated an additional transformation vector, in which betalain accumulation would be restricted to ripening fruit by driving *CYP76AD1* gene expression under the fruit-specific E8 promoter (23). Transformation of the modified pX11 (E8) vector to tomato (cv. M82; pX11-M82-E8) indeed resulted in generation of plants with betalain pigmentation restricted to the ripening fruit stage (Fig. 3A). Betacyanin concentration in juice extracted from pX11-M82-E8 fruit was determined to be $50 \pm 10 \text{ mg} \cdot \text{L}^{-1}$ (mean $\pm$ SEM), fivefold lower than in pX11-Mt fruit. Notably, none of the transgenic plants reported above displayed an apparent developmental phenotype or growth retardation, despite constitutive accumulation of betalains at high quantities.

Combining Betalain and Anthocyanin Production in Tomato. Engineering of a different class of pigments, namely anthocyanins, has been achieved previously via expression of the common snapdragon (*Antirrhinum majus*) transcription factors Delila (Del) and Rosea1 (Ros1) in tomato (24). Considering the health-beneficial qualities of betalains, anthocyanins, and carotenoids, it would be of interest to engineer edible crops that accumulate all three classes of pigments. To this end, pX11-Mt was crossed with a 35S::Del/PNH::Ros1 tomato line (in the cv. MicroTom background), which accumulates anthocyanins in fruit and vegetative tissues (25). The cross resulted in generation of plants that produce both anthocyanins and betalains, as could be seen by their distinct fruit pigmentation (Fig. 3C). MALDI mass spectrometry imaging (MALDI-MSI) was used for analyzing wild-type, pX11-Mt, Del/Ros1, and pX11 × Del/Ros1 fruit sections, together with the anthocyanidins petunidin and malvidin, and the carotenoids lycopene and phytofluene. Anthocyanidin signals were significantly higher than their respective glycosylated anthocyanin compounds, due to removal of the sugar moiety by the MALDI ionizing laser. While betanin and the identified carotenoids were generally found

to be uniformly dispersed across the section, anthocyanins accumulated mostly around the skin area (Fig. 3D and Fig. S2). In addition, we also detected chlorophyll a and naringenin chalcone pigments in fruit of all four genotypes, indicating that five different pigment classes are in fact accumulated in ripe pX11 × Del/Ros1 fruit, including carotenoids, anthocyanins, betalains, chlorophylls, and chalcones (Fig. 3D and Fig. S2).

Variation in Tobacco Flower Color Obtained by Expressing Different Combinations of Betalain Pathway Genes. All pX11-expressing plant species displayed dominant red-violet coloration due to the accumulation of high amounts of betacyanins. The pX11 vector included the *CYP76AD1* gene, encoding an enzyme catalyzing both the hydroxylation of tyrosine to L-DOPA and the conversion of L-DOPA to cyclo-DOPA. A related enzyme in red beet, *CYP76AD6*, uniquely exhibits tyrosine 3-hydroxylation activity to form L-DOPA (18). Since cyclo-DOPA derivatives are required for the formation of betacyanins, expression of *CYP76AD6* instead of *CYP76AD1* was likely to result in the formation of betaxanthins but not betacyanins, as previously observed by transient expression in *Nicotiana benthamiana* (18). To explore the possibility of generating transgenic plants that accumulate only betaxanthin-type betalains, a plant transformation vector was generated for 35S driven expression of *BvDODA1* and *CYP76AD6* (i.e., pX13; Fig. 4A). An additional vector (termed pX12), was generated for expression of both cytochrome P450 genes *CYP76AD1* and *CYP76AD6*, alongside *BvDODA1* and *cDOPA5GT* (Fig. 4A). While pX13-expressing tobacco plants (pX13-Nt) produced only betaxanthins, resulting in formation of yellow-pigmented flowers, expression of pX12 generated plants (pX12-Nt) with flowers of an orange-pink hue (Fig. 4B). LC-MS analysis of petals derived from pX11-Nt, pX12-Nt, and pX13-Nt plants indicated that the different colors observed in flowers of the three lines are the result of varying betacyanin/betaxanthin ratios; pX11-Nt flower extracts predominantly contained betacyanins, pX13-Nt contained betaxanthins only, while pX12-Nt extracts contained both classes of betalains, with a lower betacyanin/betaxanthin ratio compared with pX11-Nt flowers (Fig. 4C).

Differences in betaxanthin versus betacyanin accumulation could also be observed by imaging under blue light (Fig. 4B), in which betaxanthins have a typical fluorescence (26). The flux toward biosynthesis of red-violet betacyanins or yellow betaxanthins can thus be manipulated via expression of *CYP76AD1*,

Fig. 4. Engineering betalain production in flowers. (A) Schematic of the pX11, pX12, and pX13 over-expression vectors. nptII, kanamycin resistance marker; DODA1, *B. vulgaris* DOPA 4,5-dioxygenase; CYP76AD1/CYP76AD6, *B. vulgaris* cytochrome P450; cDOPA5GT, *Mirabilis jalapa* cyclo-DOPA-5-O-glucosyltransferase. (B) Introduction of the pX11, pX12, or pX13 vectors into tobacco results in formation of differently colored flowers, viewed from top (Top row), or magnified and viewed under bright-field (Middle row), or blue light (Bottom row), in which betacyanins are typically fluorescent. (C) LC-MS analysis of pX11, pX12, and pX13 tobacco petals. Extracted ion chromatograms (XIC) of masses ($M + H = 309.1, 331.1, 340.1, 551.1$), respectively corresponding to proline-betaxanthin, tyramine-betaxanthin, glutamine-betaxanthin, and betanin/isobetanin. Vertical axes are linked. (D) *Petunia x hybrida* (cv. Mitchell) flowers, in wild type (Left) or a betalain-producing line, following expression of the pX11 vector (Right).

CYP76AD6, or a combination of both (Fig. 1). To examine the possibility of altering flower color in a known ornamental plant, we transformed a white petunia variety (*Petunia x hybrida* cv. Mitchell) with the pX11 vector. As observed with other pX11-transformed species, petunia plants showed red-violet pigmentation in roots and vegetative tissue and produced flowers of a pale violet color (Fig. 4D). LC-MS analysis of pX11-Petunia petals validated the occurrence of betanin and isobetanin as the main betalains produced.

Betalain Production in Tobacco BY-2 Cell-Suspension Culture. Biotechnological production of betalain pigments may provide new viable sources for natural colorants in the food, pharma, and cosmetics industries. To date, most research has primarily been focused on development of *B. vulgaris* hairy root culture or cell culture for the production of betalains (16). Metabolic engineering for heterologous betalain production could enable the development of numerous new sources for these pigments. One viable source may be the culture of a well-established plant cell line such as tobacco BY-2. Expression of the pX11 and pX13 vectors in tobacco BY-2 cells resulted in the generation of red-violet or yellow cells, respectively (Fig. 5A and B). Interestingly, red and yellow pigmentation was observed within cells but not in the solid or liquid media in which they were grown, suggesting that betalains accumulate in the cells and are not secreted to the extracellular medium. LC-MS analysis of cell extract of the pX11-expressing cells showed betanin and isobetanin as the major betacyanins. Several betaxanthins were identified in both the pX11 and pX13 cell lines, including glutamine-betaxanthin and alanine-betaxanthin (Fig. 5C). Betacyanin or betaxanthin concentrations in pX11 or pX13 cell extracts were determined by spectrophotometric analysis to be $47 \pm 1 \text{ mg} \cdot \text{L}^{-1}$ and $100 \pm 7 \text{ mg} \cdot \text{L}^{-1}$ (means $\pm$ SEM), respectively.

Betalains Confer Resistance to *B. cinerea* Infection in Tobacco Leaves. It has previously been suggested that betalains may have evolved due to a presumed role in defense against pathogenic fungi (27). However, evidence for antifungal activity of betalains in scientific literature is exceedingly scarce. Heterologous betalain production in plants provides an excellent platform for studying the putative antifungal activities of betalains in planta. We therefore examined plant resistance toward *B. cinerea* infection in wild-type versus pX11-expressing tobacco plants. Droplets of *B. cinerea* spore suspension

were applied on leaves of 4-wk-old plants and the extent of *B. cinerea* infection was estimated by measurements of the lesion area around the infection points. Plants expressing pX11 exhibited a 90% reduction in average lesion area versus wild-type plants 2 d postinfection (DPI). At 3 DPI, 45% of infected wild-type leaves and 4% of infected pX11 leaves were in a state of advanced necrosis. Average lesion area was determined in the remaining leaves and found to be approximately 60% smaller in pX11 versus

Fig. 5. Production of betalains in tobacco BY-2 cells. (A) Introduction of pX11 or pX13 into cells of the tobacco BY-2 line resulted in the formation of red-violet or yellow-orange calli, respectively. (B) Betalain production is observed in pX11 and pX13 cell lines grown in cell-suspension culture. (C) LC-MS analysis of pX11 and pX13 BY-2 cells. Extracted ion chromatograms (XIC) of masses ($M + H = 283.1, 340.1, 551.1$), respectively corresponding to alanine-betaxanthin, glutamine-betaxanthin, and betanin/isobetanin. Vertical axes are linked.

wild-type leaves (Fig. 6*A* and *B*). Fungal staining with aniline blue was applied on infected leaves to examine hyphae density in the lesion area; however, no clear differences between wild-type and pX11 leaves could be observed (Fig. 6*A*). Greening and depigmentation were frequently observed around infection sites in pX11 leaves. A similar effect could also be observed following injection of 30% H₂O₂ into pX11 tobacco leaves (Fig. 6*C*).

Discussion

Betalains are highly nutritious plant pigments noted for their strong antioxidant properties (28). Here, we demonstrated that heterologous production of betalains in high quantities can be achieved in a variety of plant species, including important food crops tomato, potato, and eggplant. Betalain production not only provides attractive pigmentation but also serves to enhance nutritional quality. This is of particular interest due to the small number of betalain-producing edible plants (4) and thus the limited availability of betalains in a typical human diet. While betanin, typically accumulating in red beet, was the major betacyanin produced by engineered plants, different and unique betacyanins were identified in each of the transformed species, several of which were never reported to occur in natural betalain-producing plants.

Considering the occurrence of two classes of red or yellow betalains, we also explored the possibility of generating plants with additional colors to those observed in the pX11-expressing plants. Expression of betalain-related genes in different combinations consequently resulted in generation of tobacco plants displaying red-violet, yellow, or orange-pink pigmentation. Specifically, the ratio of betacyanin/betaxanthin production was manipulated by expression of *CYP76AD1*, *CYP76AD6*, or a combination of both genes. It is likely that an array of additional hues and colors can be obtained by regulating the ratio of expression levels between these two genes. Indeed, a variety of colors is displayed by natural betalain-producing plants such as *Bougainvillea* and *Gomphrena*, where betacyanin/betaxanthin ratios were also reported to be a major factor in determination of their inflorescence colors (29). Considering the possibility of engineering betalain production on the background of ornamental plants that have many existing varieties with diverse flower colors and patterns due to anthocyanin and/or carotenoid accumulation (e.g., petunia), a myriad of novel varieties with various flower colors can possibly be developed. The ubiquitous nature of the betalain precursor tyrosine and the relative simplicity of the

betalain biosynthetic pathway also infer the feasibility of heterologous betalain production in many plant species in addition to those presented here.

Betacyanins and anthocyanins provide a similar range of colors and are both widely used today as natural colorants in the food industry. However, while numerous edible plant sources are exploited for anthocyanin recovery, red beet remains virtually the only commercially used source for production of betacyanins as food colorants (14). Despite its high betacyanin content, red beet extract has several drawbacks as a source of food colorants: it mainly produces betanin and thus has limited color variability; it carries adverse earthy flavors, due to the occurrence of geosmin and various pyrazines; and it holds the risk of carryover of soil-borne microbes (8). There are currently no natural sources in large-scale use for production of betaxanthins as food dyes. Yellow beet, for example, is not used, likely due to co-occurring phenolics that are easily oxidized and mask the yellow hue of betaxanthins (8). Evidently, it is of interest to develop alternative sources for betalain production, and particularly betaxanthins, as current solutions for natural, yellow, water-soluble pigments for commercial use in the food industry are limited. Heterologous production of betalains may provide numerous new viable sources for these pigments, such as edible plants, plant cell cultures, and yeast fermentation. Development of one such source, namely, plant cell-suspension culture, was demonstrated here as a possibility for production of either betacyanins or betaxanthins, by expression of *CYP76AD1* or *CYP76AD6*, respectively.

Betalain-producing tobacco plants showed increased resistance toward leaf infection of the phytopathogenic “gray mold” fungus, *B. cinerea*, compared with control wild-type plants. Red depigmentation was frequently seen around the infection points, implying a possible mechanism for the increased fungal resistance, whereby betalains were degraded due to their scavenging activity of reactive oxygen species (ROS), thus delaying plant cell death and proliferation of the necrotrophic fungus. Interestingly, a similar mechanism of action was previously suggested for the increased *B. cinerea* resistance observed in transgenic anthocyanin-producing tomato fruit (30). The fact that both betalains and anthocyanins exhibit a similar function in protection against a phytopathogenic fungus contributes to the understanding of the intriguing evolutionary interplay between these two pigment classes, which are taxonomically distributed in plants in a mutually exclusive fashion. It is plausible that betalains could not have replaced anthocyanins in the Caryophyllales if they could not maintain roles other than pollinator attraction. Additional parallel physiological functions and properties of betalains and anthocyanins have been postulated (31, 32).

It will be of interest to further study the mechanism behind the antifungal activities of betalains and to examine whether heterologous betalain production can be implemented to confer resistance toward additional phytopathogens, including those infecting fruit, vegetative tissues, or roots. With the resistance they confer toward gray mold as well as their striking colors and nutritional qualities, betalain engineering holds the promise of creating new value for consumers, as well as producers and suppliers of food crops and ornamental plants.

Materials and Methods

Plant Material and Growth Conditions. *Solanum tuberosum*, *Solanum melongena*, *Solanum lycopersicum*, *Solanum nigrum*, and *Petunia × hybrida* plants were soil grown in a greenhouse with long-day light conditions (25 °C). *N. tabacum* plants used for *B. cinerea* infection were soil grown in climate rooms (22 °C; 70% humidity; 18/6 h of light/dark).

Plant Transformation and Regeneration. Agrobacteria-mediated plant transformation and regeneration was done according to the following methods: tobacco leaf discs (33), eggplant (34), tomato (35), potato (36), petunia (37), *S. nigrum* (18), and BY-2 cells (38). All plant species were transformed using agrobacteria GV3101 strain. Plant tissue culture and BY-2 cell suspension culture was carried out in climate rooms (22 °C; 70% humidity; 16/8 h of light/dark, 2,500 Lux light intensity). Tomato pX11 transformation was done in the cv. MicroTom background. The pX11 lines were crossed with *S. lycopersicum*

Fig. 6. Gray mold resistance in tobacco plants engineered for betalain production. (*A*, Top row) Leaves of wild-type and pX11 tobacco plants were infected with droplets of *B. cinerea* conidial suspension and photographed 3 d postinfection. (Bottom row) Fungal staining with aniline blue, 3 d postinfection. (*B*) Wild-type and pX11 plants infected with a total of 500 *B. cinerea* spores per plant were scored for lesion size 2 and 3 d postinfection. Average lesion areas are shown with error bars representing SEM. ****P* value < 0.001 (*t* test). (*C*) Red depigmentation was frequently detected around *B. cinerea* infection points in pX11 tobacco leaves (Left), similarly to the observed effect following injection of 30% H₂O₂ (Right).

cv. M82 (SP⁺) and with Del/Ros1 (in the cv. MicroTom background). Both crosses were obtained by transferring pX11 pollen to flowers of the respective plants.

Generation of DNA Constructs. Gene sequences used in this study were *cDOPA5GT* (GenBank accession AB182643.1), *BvDODA1* (HQ656027.1), *CYP76AD1* (HQ656023.1), and *CYP76AD6* (KT962274). Additional information is given in *SI Materials and Methods*.

LC-MS Analysis. Samples were analyzed using a high-resolution UPLC/PDA-qTOF system comprised of a UPLC (Waters Acquity) connected on-line to an Acquity PDA detector (200–700 nm) and a qTOF detector (tandem quadrupole/time-of-flight mass spectrometer, XEVO, Waters) equipped with an electrospray ionization (ESI) source. Additional information on betalain extraction and LC-MS analysis of betalains is provided in *SI Materials and Methods*.

MALDI Mass-Spectrometry Imaging Analysis. MSI measurements were performed using a 7T Solarix FT-ICR (Fourier transform ion cyclotron resonance) mass spectrometer (Bruker Daltonics). Additional information on MALDI-MSI is given in *SI Materials and Methods*.

Spectrophotometric Quantification. Betacyanin or betaxanthin content of all samples was assessed by measuring absorption at 535 nm or 475 nm, respectively, subtracting the value of absorption at 600 nm, and calculated using a previously described method, applying a molar extinction coefficient

of $\varepsilon = 60,000 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for betacyanins and $\varepsilon = 48,000 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for betaxanthins (39). Samples were diluted in double-distilled water (DDW) to obtain solutions of $\text{OD}_{535} < 2.0$. Concentrations are given in milligrams/kilograms where extraction solvent was added to sample and in milligrams/liters for samples to which no extraction solvent was added.

Tobacco *B. cinerea* Infection. Wild-type and pX11-expressing tobacco plants were screened by a leaf infection assay as previously described (40). Detailed information on infection experiments and analysis is provided in *SI Materials and Methods*.

TEAC Assay. The total antioxidant capacity of wild-type and betalain-producing tomato was measured by the TEAC assay (41). Whole tomato fruit were ground. Following centrifugation, supernatant was used for the assay without addition of solvent. TEAC was measured by 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS^{•+}) decolorization using preformed ABTS^{•+} radical cation, and calculated by absorbance at 734 nm, in comparison with Trolox, as described previously (42). All determinations were performed in triplicates, where each biological sample is a pool of five tomato fruit.

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