Trends in Plant Science

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Opinion

Capsaicinoids: Pungency beyond *Capsicum*

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Capsaicinoids are metabolites responsible for the appealing pungency of *Capsicum* (chili pepper) species. The completion of the *Capsicum annuum* genome has sparked new interest into the development of biotechnological applications involving the manipulation of pungency levels. Pungent dishes are already part of the traditional cuisine in many countries, and numerous health benefits and industrial applications are associated to capsaicinoids. This raises the question of how to successfully produce more capsaicinoids, whose biosynthesis is strongly influenced by genotype–environment interactions in fruits of *Capsicum*. In this Opinion article we propose that activating the capsaicinoid biosynthetic pathway in a more amenable species such as tomato could be the next step in the fascinating story of pungent crops.

Highlights

Capsaicinoids are secondary metabolites that confer *Capsicum* ('chili' peppers) fruits their appealing pungency ('heat') and have multiple proven health benefits and industrial applications.

Five *Capsicum* species have been domesticated. However, even in fruits of the same species, pungency is not consistent owing to the strong influence of the environment on capsaicinoid levels. Furthermore, multiple agronomic issues are prevalent in chili pepper cultivation, such as wilting in hot days, flower drop, and virus susceptibility.

Capsicum belongs to the Solanaceae family, and recent completion of its full genome sequence shows that inactive genes for the biosynthetic pathway of capsaicinoids are also present in tomato. We propose that the use of state-of-the-art genome engineering techniques to activate capsaicinoid biosynthesis in tomato is a suitable option to exploit these valuable secondary compounds.

The Nutritional Value of Capsaicinoids

The momentous voyage of Christopher Columbus in search of a new route to India was driven by the European demand for spices – mainly black pepper (*Piper nigrum* L.), which at the time fetched prices similar to gold [1,2]. Instead, Columbus arrived in Central America and brought back a species that would quickly become 'the world's most popular condiment': chili peppers of the *Capsicum* genus [3]. The genus comprises more than 30 species, with only five domesticated crops, namely the closely related *C. annuum*, *C. chinense*, and *C. frutescens*, as well as *C. baccatum* and *C. pubescens* [4], which display a broad variety of fruit size, shape, color, and **pungency** (see Glossary; Figure 1). The appealing pungency ('heat' or 'bite'; Box 1) of hot chilies is produced by the accumulation of a group of alkaloids, called capsaicinoids, whose major representatives are capsaicin and dihydrocapsaicin [5]. These secondary metabolites are produced through a complex series of reactions involving the convergence of the phenylpropanoid and branched-chain fatty acid pathways (Figure 2) [6].

In the context of the debate on food security, nutritional and nutraceutical properties are quickly gaining importance alongside crop yield as breeding targets [7,8]. Plants represent a fundamental reservoir of chemical compounds beneficial to human health, mainly derived from their extraordinarily rich **secondary metabolism** [9,10]. However, breeding efforts mostly focused on attaining high yields have resulted in a serious nutritional decline of grains, fruits, and vegetables [11]. Finding sustainable pathways to boost the production of vitamins and secondary metabolites such as polyphenols and carotenoids in crops is thus a top priority in the plant research agenda [12,13]. *Capsicum* fruits contain high levels of antioxidants such as vitamins A, C, and flavonoids [14]. Hot peppers also produce variable amounts of capsaicinoids, which have demonstrated anti-inflammatory [15], antioxidant [16], antitumoral [17], and weight-loss properties [18]. All of these effects suggest that a regular intake of capsaicinoids is beneficial for health and that, therefore, hot peppers can be considered to be **functional foods** [19]. Furthermore, topical capsaicin applications are an effective pain-management treatment with low risk for adverse drug–drug interactions [20]. Remarkably, only *Capsicum* fruits contain these appealing compounds. We next examine their physiological roles in plants.

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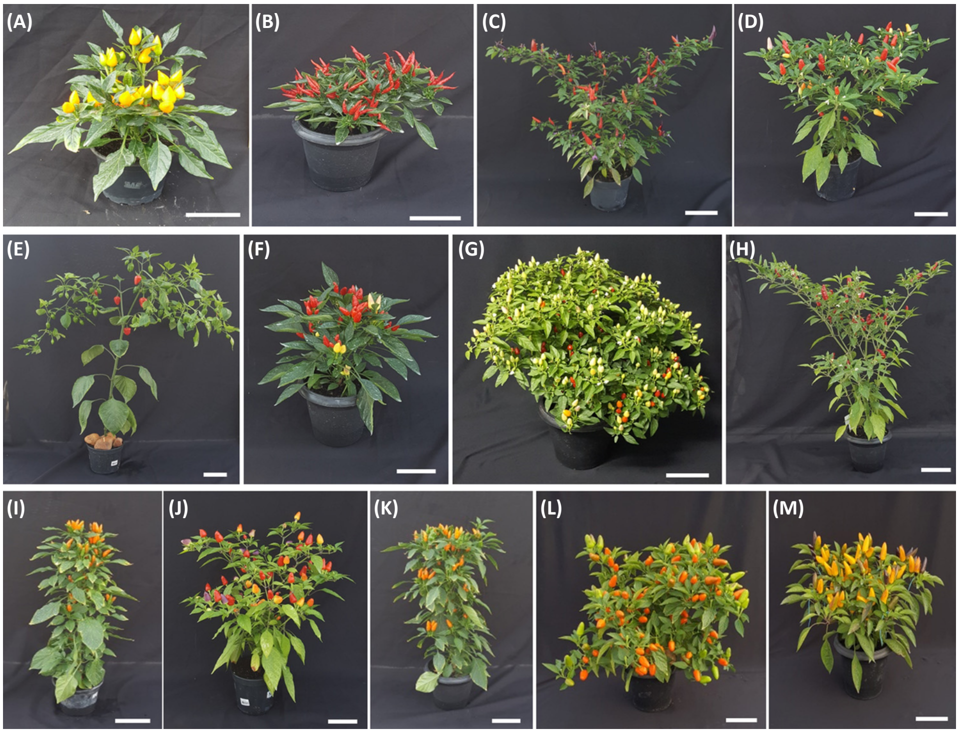

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Figure 1. Variation in Plant and Fruit Morphology in the *Capsicum* genus. Accessions of *Capsicum annuum* (A–M) and *Capsicum chinense* (N). Scale bar, 10 cm.

The How and Why of Pungency in *Capsicum*

Pungency and Seed Dispersal

The development of nutritious and highly caloric fruits to enlist efficient agents of seed dispersal presented plants with the conundrum of avoiding seed predators [21]. How a fruiting plant can repel inefficient dispersal agents and favor more effective ones is a long-standing question in angiosperm ecology [22]. One possible solution is the production of secondary metabolites, which are thus abundant in fleshy fruits [23,24]. The **directed deterrence hypothesis** (DDH) posits that fruit secondary metabolites can selectively repel seed predators without deterring seed dispersers [25,26]. There is mounting evidence that secondary metabolites have indeed been instrumental in shifting plant–animal relationships from **predation** to **mutualism** [27,28]. Experiments with captive animals showed that capsaicinoids in hot chili fruits were irritant to mammals but not to birds. The basis for this selective toxicity of capsaicinoids was eventually demonstrated at the molecular level. The vanilloid receptor 1 (VR1), a cation channel on sensory

Box 1. The Measure of Chili Pungency

The most widely used measure of chili pepper pungency is the ‘Scoville scale’, after its inventor, Wilbur Scoville, who in 1912 created the Scoville organoleptic test [116]. Volunteers test serial dilutions of pepper extract, and the degree of dilution is translated into Scoville heat units (SHU). The scale remains popular although it is unreliable and subjective; in modern times has been superseded by the use of HPLC [117]. Pungency units are thus defined as parts of capsaicin per million parts of pepper fruit dry mass. Pungency units can be converted to SHU by multiplying by 16 (e.g., pure capsaicin is 16M SHU, pepper spray is 5M SHU). Some of the hottest chili pepper varieties can exceed 1M SHU, and have fanciful names such as Scorpion, Carolina Reaper, or Dragon’s Breath.

Glossary

Biofactory: a biological system used to produce useful amounts of chemical compounds. Many organisms are ‘customized’ (through selection or genetic manipulation) as production platforms for the manufacture of biopharmaceuticals such as enzymes, recombinant proteins (in *E. coli* or yeast), vaccines, and antibodies (generally in plants).

Capsinoids: compounds that are structurally similar to capsaicinoids, but that lack the pungent taste. The main capsinoids found in chili peppers are capsiate, dihydrocapsiate, and nordihydrocapsiate. Many of the health benefits described for capsaicinoids have also been attributed to capsinoids. Thus, manipulation of the capsinoid pathway is also an appealing prospect given that a segment of the population cannot tolerate the pungent flavor.

Cisgenesis: a form of genetic engineering whereby a crop plant is genetically modified using DNA fragments contained in the species itself or in closely related, interfertile species.

Directed deterrence hypothesis (DDH): first proposed by Cipollini and Levey in 1997 [25], the hypothesis states that plants produce secondary metabolites that selectively preclude foraging by specific animals (by being toxic or unpalatable), but not by others that can then act as efficient vectors for seed dispersal.

Exaptation: a shift in the function of a trait during evolution. In the case of capsaicinoids, it is possible that their original function was as protective agents for the plants against attack by pathogens, and subsequently they acquired a role as deterrents of fruit-eating mammals.

Functional foods: whole, enhanced, or fortified foods containing ingredients that aid specific body functions for health benefits (beyond basic nutrition).

Knock-in: targeted insertion of a DNA sequence that is integrated into a specific genomic locus.

Mutualism: a cooperative relationship between two organisms whereby both benefit from the interaction.

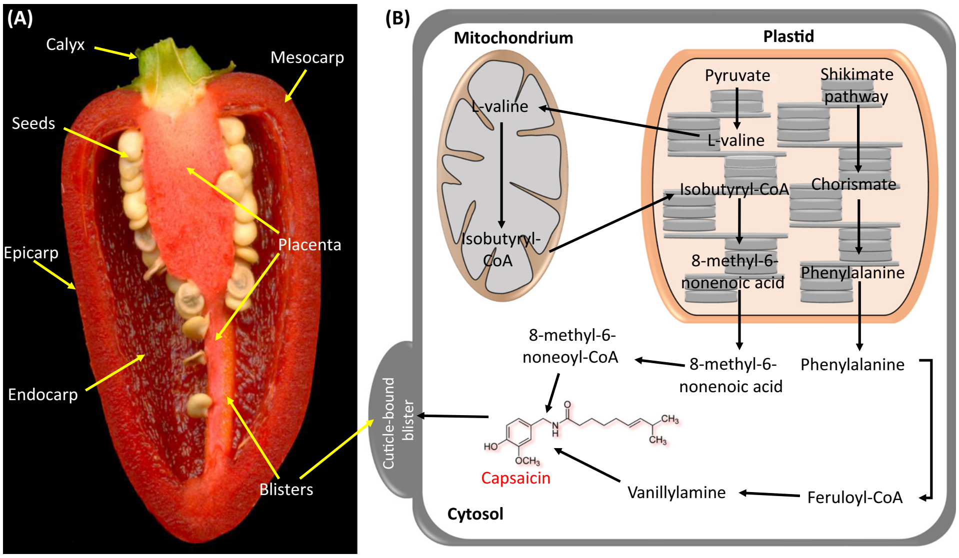

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Figure 2. Capsaicinoid Biosynthetic Pathway. (A) Cross-section of chili pepper fruit. (B) Schematic representations of the subcellular locations of the main steps involved in capsaicinoid biosynthesis. The convergence of two pathways is required – the phenylpropanoid pathway, which leads formation of vanillylamine, and the branched chain fatty acid pathway, which forms the 8-methyl-6-nonenoyl-CoA; the two final compounds are condensed by capsaicinoid synthase to form capsaicin. Abbreviations: ACS, acyl-CoA synthetase; AMT, aminotransferase; BCAT, branched-chain amino acid aminotransferase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; COMT, caffeoyl-CoA 3-*O*-methyltransferase; CS, capsaicinoid synthase; FatA, acyl-ACP thioesterase; HCHL, hydroxycinnamoyl-CoA hydratase lyase; PAL, phenylalanine ammonia-lyase. Redrawn from Mazourek *et al.* [6] and Kim *et al.* [95].

nerve endings which responds to noxious thermal stimuli, is activated by capsaicin in mice [29]. Cloning and functional characterization of the chicken VR1 ortholog showed that, in birds, it fails to be activated by capsaicin owing to differences in the amino acid sequence at key positions that affect ligand binding [30].

The evolutionary emergence of pungency is considered to be a derived trait within the *Capsicum* genus, and is not found in species of the Andean clade, the most ancient group of *Capsicum* species [31]. However, it is not known for certain why pungency has been fixed in some species of the genus and not in others. It could be the result of an ecological trade-off which allows the biosynthetic pathway to remain active [32]. Pungency precludes fruit consumption by small mammals, to avoid loss of seed viability in their digestive tract, and instead favors consumption by birds, which can provide seed dispersion over greater distances [33]. Ironically, the compounds produced to deter mammal consumption were instrumental in the formation of a reciprocal relationship with mammals that helped *Capsicum* to spread to all continents: humans [34]. Seeds of pungent fruits are also less prone to infection by the fungal pathogen *Fusarium*, and thus pungent individuals propagate their offspring more efficiently than non-pungent ones [35]. It is possible that this was the original function of capsaicinoids and that the selective deterrence of mammals was an **exaptation** occurring later during the evolution of *Capsicum* species [36].

Challenges of Producing *Capsicum* Fruits with Stable Pungency Levels

Growth and Cultivation of *Capsicum* Species

Unlike some of its domesticated counterparts in the Solanaceae family (tomato, *Solanum lycopersicum*; potato, *S. tuberosum*; tobacco, *Nicotiana tabacum*), *Capsicum* species are

Predation: an interaction between two organisms in which one (the predator) kills and feeds off the other (the prey).

Pungency: technical term for food commonly referred to as 'spicy' or 'hot'.

Secondary metabolism: pathways and chemical products of metabolism with specific functions that are generally associated with plant–environment interactions, including stress responses. Humans use a myriad of secondary metabolites for a variety of purposes, for instance alkaloids (nicotine, caffeine, morphine), terpenes (artemisin, limonene, pinene) and flavonoids (hesperidin, rutin, quercetin).

Synteny: conservation in the relative position of genetic loci within a chromosome among different species. A powerful tool in comparative genomics that is used to infer gene function and phylogenetic relationships between species.

notoriously labor-intensive and difficult to cultivate crops. Pungent varieties are predominantly cultivated in the open field, and are thus vulnerable to environmental conditions detrimental to fruit yield. High air temperature, high CO_2 concentration, and excess precipitation can all retard growth and increase the incidence of disease [37]. Seed germination can be very slow and uneven depending on species, cultivar, and post-harvest handling [38,39]. Seed viability is affected by the maturity of fruits, and can be reduced considerably if they are harvested either too early or too late [40].

Soil-borne diseases and nematodes are prevalent in *Capsicum*, and grafting onto disease-resistant rootstocks is a common, although expensive and time-consuming, cultural practice [41]. *Capsicum* yield is poorly responsive to N and K supply because most of the effect is translated into vegetative instead of reproductive growth [42,43]. Fruit set can be erratic owing to flower drop induced by excessive N fertilization, high wind, and lack of pollination [44]. *Capsicum* species are self-pollinating, but their floral structure readily allows cross-pollination (mostly by insects) [45], making the production of pure seed cumbersome and costly [46]. Even if all these agronomic shortcomings can be resolved satisfactorily, the highly variable nature of capsaicinoid biosynthesis, which is also under strong environmental control, represents one further hurdle towards the production of consistent levels of pungency in fruits [47].

Capsicum Breeding and Biotechnology

Varieties with more consistent pungency levels could be produced by either breeding or biotechnological manipulation. Classical breeding programs have contributed to the creation of elite *Capsicum* varieties, although numerous interspecific crossing barriers are present within the genus that prevent the full exploitation of favorable traits [48]. The alternative is to manipulate traits of interest by *Agrobacterium*-mediated genetic transformation [49]. *Capsicum* species, however, are notoriously recalcitrant to transformation by *Agrobacterium* [50]. *In vitro* regeneration capacity is not an obstacle, and protocols can be adjusted for each particular genotype of interest [51]. The limiting step appears to be the introduction of exogenous DNA into the plant. Multiple factors influence the process of DNA transfer and integration into the plant genome, many of which are not fully understood [52]. Currently available *Capsicum* transformation protocols have low efficiency, poor reproducibility, and high genotype-dependence [53,54]. Thus, biotechnological manipulation of capsaicinoids in *Capsicum* is not a suitable option in the toolbox of the breeder.

The Dynamics of Capsaicinoid Accumulation in Fruits

Capsaicinoid levels are highly dynamic during fruit development. Their levels appear to be influenced by the ontogenetic trajectory of the fruit. Capsaicinoids begin to accumulate from the early stages (10 days after pollination, DAP) of fruit development, peak at around 40 DAP, and then decrease sharply [55–58]. The late decrease in capsaicinoid content appears to result from high peroxidase activity which oxidizes capsaicinoids in the presence of H_2O_2 [59,60]. Therefore, the final amount of capsaicinoids in the fruit depends on a complex balance between the factors that stimulate their synthesis and degradation. Capsaicinoid levels are thus highly plastic and are most likely determined by a complex set of ecological and physiological trade-offs with strong input from the environment.

Light

Light stimulates capsaicinoid formation in fruits harvested immature and kept under artificial lighting [61]. Moreover, ripe pepper fruits maintained under a fluorescent light contained twice the capsaicin content of peppers stored in darkness [62]. Keeping fruits constantly illuminated at moderate irradiance (150–350 μmol m^{-2} s^{-1}) was found to be more inductive of capsaicinoid biosynthesis than maintaining plants at high luminous intensities in the greenhouse during the summer season [63]. This suggests that photoperiod is seemingly more important to capsaicin accumulation than light intensity *per se*. Light has a positive influence on the expression of the *capsaicin synthase* gene (*CS,* discussed further below), which harbors light-responsive motifs in its promoter region [64], *KAS* (*keto-acyl ACP synthase*) and *AMT* (*aminotransferase*) [65], and a negative effect through the induction of peroxidases that degrade capsaicin [60]. It is hitherto unknown how this balance is controlled and fine-tuned.

Temperature

Pungency level is highly variable in response to air temperature between cultivars and species [66], and therefore there is no consensus regarding the temperature effects on capsaicinoid synthesis and accumulation. For some varieties, higher temperature favors the accumulation of total capsaicinoids, while in others the accumulation of capsaicinoids is affected negatively. It has been hypothesized that respiration might play an important role in temperature responses by altering the consumption of photoassimilates and carbon partitioning between plant growth, lignin, and capsaicinoid synthesis [67]. Furthermore, high night-time temperatures can have a greater impact on capsaicin contents than daylight temperatures [63]. The *CS* gene promoter region contains heat stress consensus elements (HSEs) which could possibly stimulate the synthesis of capsaicinoids in response to high temperature [64].

Water

In general, water stress reduces fruit yield and increases capsaicinoid accumulation in hot pepper, albeit in a strongly variety-dependent manner [68]. Water stress decreases fruit size compared to well-watered plants, but affect less the formation of placental tissue, leading to a higher proportion of placental tissue per fruit [69]. This is consistent with a higher production of capsaicinoids because the placenta is their site of synthesis and accumulation. This effect, however, is also cultivar-dependent [70]. Drought increases the activity of enzymes necessary for capsaicin biosynthesis (PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; and CS) and reduces capsaicin degradation by peroxidases [69,71]. By contrast, the proportion of pungent plants in wild *Capsicum* populations native from Bolivia increases under higher moisture [32]. In this case, pungency could be beneficial because capsaicinoids protect the fruit from pathogens, especially from fungi that thrive under high-moisture conditions. Thus, pungency is increased by both water shortage and excess, but for different ecological and physiological reasons.

Mineral Nutrition

Mineral nutrition influences the metabolism of capsaicinoids. In this sense, N and K are the main players. N availability directly affects capsaicin accumulation because the synthesis of a single capsaicin molecule involves three amino acids – phenylalanine, valine, and leucine – and the still unidentified amino donor required for the formation of vanillylamine (Figure 2). The capsaicinoid content in *Capsicum* fruits has been associated positively with available N in the soil [42,43]. Similarly, high nitrate nutrition enhanced capsaicin levels in placenta from *Capsicum* fruits cultured *in vitro* [72] or harvested from hydroponic plants [73]. *C. annuum* fruits from plants grown under different concentrations of K did not show alterations in capsaicinoid accumulation [42]. By contrast, even though K does not participate in capsaicinoid metabolism, an increase in K application resulted in a significant decrease of capsaicin levels and leaf N content in *C. chinense* [43]; thus, the level of K levels might indirectly affect capsaicin accumulation through its effects on fruit development [74].

Environmental factors have complex and sometimes contrasting effects on pungency levels (Figure 3). Genotypes, either species or cultivars, are highly variable in their response to specific environmental conditions. Analysis of 49 accessions of *C. chinense*, collected in different regions of Brazil, revealed wide variation in metabolic parameters [75]. Similar results were found for 32 accessions of *Capsicum* with respect to productivity, fruit pigments and morphology, capsaicinoids, **capsinoids**, and vitamins C and E [24]. The relative scarcity of data in this area makes it difficult to draw definitive conclusions. Studies harnessing the abundant natural variation in the genus, associated with high-throughput genotyping, may shed more light on this interesting topic [76–78].

Activating Pungency Biosynthesis in Tomato Fruits: Towards ‘Hot’ Tomatoes

The *Capsicum* and tomato clades split at least 19 Mya ago [79]. The genomes of *Capsicum* and tomato are well preserved, with a basic chromosome number of $x = 12$ in both species and major conserved **syntenic** segments between them [80]. *Capsicum* fruits are generally considered non-climacteric; however, the physiological and morphological events during fruit-set are similar to those in tomato [81,82]. Yields of hot pepper seldom exceed 3 tons per hectare on the field in about 4–5 months of culture [83]. For tomato, on the other hand, it is not uncommon to reach 110 t/ha on the field over a 120 day cropping cycle [84]. In addition to being one of the main horticultural crops in the world, tomato is a well-established model species [85] that is highly amenable to biotechnological manipulation [86], and there are miniature model cultivars, such as Micro-Tom, that can be grown quickly and in large numbers in reduced space [87]. Thus, engineering tomato into a capsaicinoid **biofactory** is a conceptually promising approach for sustainable production of these secondary compounds [88,89].

Capsaicinoids consist of at least 23 different chemical species [90], of which capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide) together account for $\sim$90% of the total fruit capsaicinoid content [56]. Capsaicinoids are

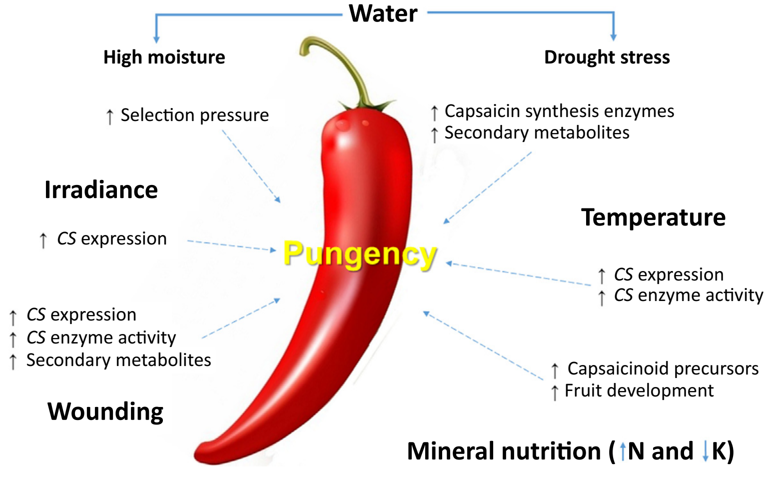

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Figure 3. Some Factors Directly or Indirectly Influencing Pungency Levels in *Capsicum*. The expression of the gene (*CS*) encoding capsaicinoid synthase is directly affected by irradiance, temperature, and wounding. Higher temperatures and wounding also increase this enzyme activity. The supply of N directly influences the capsaicin pathway because this element is directly related to the production of the precursor molecules of capsaicin. K influences development of the fruit, and thus indirectly influences the size of the placenta by allowing the production of larger fruits. The scheme does not apply to all varieties in all situations but presents the standard behavior in most cases.

synthesized in the fruit placenta through the integration of two different biochemical pathways that involve multiple intracellular compartments (Figure 2). Using phenylalanine as a substrate, the phenylpropanoid pathway provides vanillylamine, whereas the branched-chain fatty acid pathway produces 8-methyl nonenoic acid from valine. The enzyme CS, also known as PUN1, combines both compounds to produce capsaicin and, by condensing vanillylamine with different fatty acid substrates, other capsaicinoids [91,92]. The intracellular location and molecular mechanism of this last step is not yet clear, although it appears to be located in the cytosol. Tomato has an active phenylpropanoid pathway in the fruit peel that can be upregulated by overexpression of specific transcription factors [93]. Although valine is found in the tomato fruit, the intermediary compounds in the branched-chain fatty acid pathway have not hitherto been described, either because of technical limitations or because their levels are below the level of detection with currently available methods [94].

Comparison of the *Capsicum* genome to that of tomato has revealed the presence of all the necessary genes for capsaicinoid production in the tomato genome [82,95]. Genes of the capsaicinoid biosynthetic pathway have been analyzed in both species to better understand the absence of pungency in tomato. In sweet peppers, loss of pungency can occur through a variety of different mutations that affect gene expression in the pathway [96,97]. Pungency, therefore, appears to be under transcriptional control, as borne out by the higher expression levels of capsaicinoid biosynthesis genes in the placental septum of pungent cultivars [58,98]. Compared to chili peppers, in tomato some genes have lower levels of expression (*PAL*; *C4H*; *ACL*, *acyl carrier protein*, and *AMT*, *aminotransferase*); some have lower expression levels with temporally restricted expression (*COMT*, *caffeoyl-CoA 3-O-methyltransferase*; and *FaTA*, *acyl-ACP thioesterase*), in addition to *KAS*; and two genes are not expressed at all (*BCAT*, *branched-chain amino acid aminotransferase*, and *CS*) [95].

Two genome-engineering strategies could be used in tandem to activate capsaicinoid biosynthesis in the tomato (Figure 4, Key Figure). One is the use of transcriptional activator-like effectors (TALEs), a suite of proteins secreted by pathogenic *Xanthomonas* spp. bacteria when they infect plant hosts [99]. Tools for robust transcriptional activation of endogenous genes exploiting customized TALEs have recently been developed that allowed multiplex activation of up to four genes in plants [100]. Rapid assembly of the TALE genes into a single T-DNA vector is possible using a MultiSite Gateway recombination system [101,102] and would allow simultaneous upregulation of the expression of *PAL*, *KAS*, *COMT*, and *FaTA*. Interestingly, recent work has shown that a CaMYB31 transcription factor of the R2R3–MYB family regulates positively the transcription of multiple key capsaicinoid biosynthesis genes (*C4H*, *COMT*, *Kas*, and *AMT*) [65]. It remains to be determined whether the transcript levels achieved will be sufficient for the capsaicinoid pathway to be functional because expression could either be to low, and thus insufficient to activate the capsaicinoid pathway, or too high, which could trigger gene silencing [100].

The second strategy is the use of genome engineering for targeted replacement of promoters. The feasibility of this method has been demonstrated in tomato using a constitutive 35S promoter inserted upstream of the *ANT1* gene, which encodes an R2R3–MYB transcription factor regulating anthocyanin biosynthesis [103]. Promoter regions of the inactive genes in the capsaicinoid pathway (*BCAT*, *CS*), could be replaced with endogenous tomato fruit-specific promoters, such as *E8* [104], to produce **cisgenic** plants with transcriptionally active genes [105,106]. It remains to be determined, however, if the products of these genes are fully functional, biochemically active, and catalyze the appropriate reactions.

Key Figure

How To Make a Hot Tomato

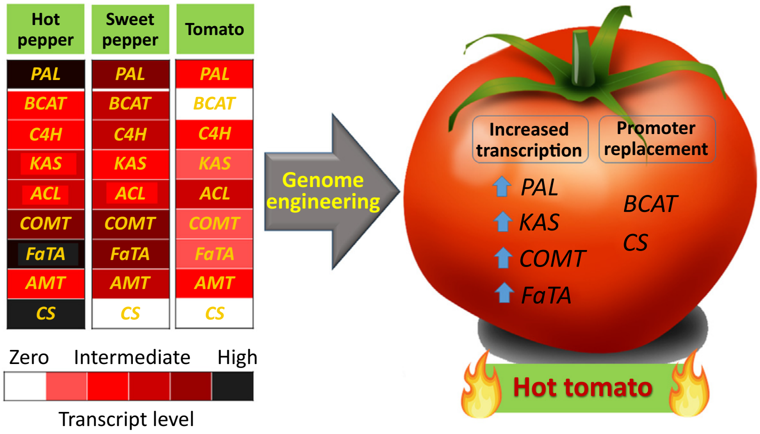

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Figure 4. Transcriptional profile of genes related to the metabolism of pungency in hot pepper, sweet pepper, and tomato (adapted from [14]). The relative values are the highest measured over all the course of fruit development. Abbreviations: PAL, phenylalanine ammonia-lyase; BCAT, branched-chain amino acid aminotransferase; C4H, cinnamate 4-hydroxylase; COMT, caffeoyl-CoA 3-O-methyltransferase; AMT, aminotransferase; FatA, acyl-ACP thioesterase; CS, capsaicinoid synthase; KAS, keto-acyl ACP synthase; ACL, acyl carrier protein. Pungency in tomato could be achieved increasing the expression of *PAL*, *KAS*, *COMT*, and *FaTA*, and by activating the expression of *BCAT* and *CS* using state-of-the-art genome engineering techniques.

Technical Challenges of Engineering Capsaicin Biosynthesis in Tomato

The most frequently described type of genome modification up to date is the creation of loss-of-function alleles using TALENs or CRISPR/Cas9 [107]. Three recently published studies have used the latter to perform genome editing of wild relatives of the tomato by multiplex editing of a suite of genes to improve agronomic performance [108–110]. While this type of modification occurs through the pathway of non-homologous end-joining for DNA repair in somatic cells, more precise modifications such as base substitutions or targeted insertions require the use of homology-directed repair. This involves further technical challenges because sequence-specific nucleases induce double-strand breaks in a specific sequence while a DNA donor template is provided for homologous recombination repair to insert the desired alteration. Homology-directed repair is not the preferred type of repair in plant cells, but efficient methods to create **knock-ins** are still forthcoming, although successful integration of promoters [103] or larger DNA sequences, including entire genes [111,112], has been reported.

The creation of novel ‘expression alleles’, namely gene variants with differential transcription compared to the original allele, can be achieved by two alternative approaches. The first is the use of TALE or CRISPR/Cas9 transcriptional activators, which have been successfully applied in *N. benthamiana*, *Arabidopsis thaliana*, and tomato [100,113]. The second, described above, is the targeted insertion of a promoter sequence upstream of the gene of interest [114]. This requires *a priori* knowledge of the spatial and temporal expression profiles produced by specific

Box 2. Production of Other Valuable Metabolites in Tomato

Successful implementation of the approach proposed here would represent an important proof-of-concept that could be extended to the production of other valuable metabolites in tomato in the future. Other pathways that could potentially be manipulated using this approach include production of the food and cosmetic pigment bixin (annatto). Bixin is the second most important natural color additive used in industry and it is produced by a single tropical plant species, *Bixa orellana* [118]. Three *B. orellana* genes encode the enzymes necessary to convert lycopene into bixin: carotenoid cleavage dioxygenase 1 (CCD1) or lycopene cleavage dioxygenase (LCD), bixin aldehyde dehydrogenase (BADH), and norbixin methyltransferase (BMT). Because tomato has a high fruit lycopene content and also harbors orthologs of those genes, their manipulation might allow bixin production in a species that is cultivated worldwide with improved agronomic practices. Alternatively, editing of the tomato *lycopene β-cyclase* gene (*SlCyc-b*) could improve β-carotene (provitamin A) accumulation at expense of lycopene (and vice versa) [109]. β-Carotene is a powerful antioxidant that is readily available in large amounts in sweet potato (*Ipomoea batatas*), winter squash (*Cucurbita pepo*), and carrot (*Daucus carota*). However, 45% of the population carry a genetic mutation that reduces their ability to convert β-carotene into the more active retinol (vitamin A) [119,120]. Tomatoes with massively increased levels of β-carotene (with subsequent cleavage into vitamin A) would therefore represent a valuable addition to their diet.

promoter sequences and also knowledge of the desired expression pattern. Some further challenges to be addressed include the expression levels that can be achieved, in particular the coordination of expression between genes, both at the spatial (tissue specificity) and temporal (developmental stage) levels, as well as the subcellular localization of the resulting gene products. These hurdles, however, are not insurmountable if a systems-biology approach is employed, integrating knowledge from multiple research areas [115].

Concluding Remarks and Future Perspectives

Capsaicinoids are the secondary metabolites that are responsible for the appealing pungency of hot peppers, a central element in the culinary activities of many countries. Their multiple health benefits make capsaicinoids a valuable nutraceutical product, which is likely to increase in demand in the coming years. Given the notorious challenges of producing stable yields of *Capsicum* with consistent levels of pungency in the fruit, we are here proposing, and working towards, an alternative approach by harnessing recent advances in genome engineering. The completion of the *Capsicum* genome, and the discovery that the tomato contains the full suite of genes necessary for pungency, paves the way for the production of 'hot tomatoes' as capsaicinoid biofactories through targeted genome manipulation. The convergence of basic knowledge with the rapidly growing repertoire of genome-manipulation tools should pave the way for engineering this and other more imaginative biosynthetic pathways (Box 2) using the tomato fruit as a chassis [88]. Our approach could provide not only new basic knowledge to help to elucidate the capsaicinoid biosynthetic pathway but also to understand its evolution and divergence in different Solanaceae species. Furthermore, it could allow the development of new functional foods appealing to a wide range of consumers (also see Outstanding Questions).

Outstanding Questions

Given that other species of Solanaceae, such as tomato, harbor the biosynthetic pathway for capsaicinoids, what is the evolutionary explanation for the restriction of pungency to species of the *Capsicum* genus?

Could capsaicinoid biosynthesis be activated specifically in the tomato fruit using state-of-the-art genome-engineering platforms?

Would the novel genomically engineered 'hot tomatoes' yield relevant and stable amounts of capsaicinoids under different environmental conditions and cultural practices?

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