Hot Tomato: Complementation of the Capsaicin Biosynthetic Pathway to Engineer Spicy Tomatoes

Summary

This project was aimed towards generating a tomato that produces capsaicin, the compound responsible for the spicy flavour of chili peppers. We identified missing genes in tomato for the capsaicin biosynthetic pathway by comparative bioinformatic analyses. From our analysis, eight genes were selected as candidates to complete the capsaicin pathway in tomato. These eight genes were all successfully cloned and placed into single and multi-gene transformation constructs. Due to unforeseen difficulties with transient tomato transformation, we opted to test gene constructs in tobacco (*Nicotiana benthamiana*). Thus far, three constructs each containing multiple genes for the capsaicin have been infiltrated by *Agrobacterium tumefaciens* into *N. benthamiana*. Four capsaicin pathway genes have been successfully co-expressed in *N. benthamiana* lines which were subsequently screened for capsaicin accumulation via spectroscopy. Several lines absorbed the wavelength for capsaicin (280nm), suggesting capsaicin accumulation, but the results were not fully clear whether they are producing it definitively. Future work will involve more powerful capsaicin detection methods (Liquid/Gas Chromatography – Mass Spectroscopy) and stable tomato transformation with all eight pepper genes to produce tomatoes with heritable spiciness.

Report and Outcomes:

The spicy flavour of chili pepper

Chili pepper (*Capsicum* spp.) is the most cultivated spice crop in the world with an annual value over $20B (USD). The spicy flavour of chili peppers is due to the accumulation of capsaicinoids, a group of alkaloids that are unique to the *Capsicum* genus and are produced exclusively in the placental tissue of chili pepper fruit (Nelson and Dawson, 1923). Capsaicinoids are the active ingredients in many pharmaceuticals, such as analgesic liniments that can treat arthritis, joint and other pain and are widely used as the active chemical agents in personal defence aerosols (*i.e.*, pepper spray).

The primary function of capsaicin is to dissuade mammalian predation and promote avian seed dispersal. Mammalian digestive tracts render chili pepper seeds nonviable, whereas the avian

gastrointestinal tract chemically and physically softens chili pepper seeds which then non-destructively boosts germination (Bosland and Votava, 2012). Most Solanaceous species possess sufficient alkaloids to cause toxicity to many mammals, partially circumventing predation, but chili peppers possess none in their tissues. Without toxic alkaloids, chili peppers evolved capsaicin as a different strategy to deter frugivory from mammals by causing a ferocious burning sensation when eaten. It appears, however, that humans are one mammal which enjoys eating it.

Producing a spicy tomato

All capsaicinoids contain an aromatic vanillyl ring derived from the amino acid, phenylalanine, connected to a branched-chain fatty acid moiety derived from the non-polar amino acids, valine, Leucine or Isoleucine. Despite the importance of capsaicin, most of the enzymes in the pathway are poorly characterised and the regulation of the pathway is unknown. Interestingly, all the orthologous genes for the genes of the capsaicin pathway are present in the tomato genome (*Solanum lycopersicum*) and other Solanaceous plants but are not expressed at the same stages in fruit development as in chili pepper. This project aimed to utilise synthetic biology approaches to overexpress enzymes from chili pepper to complement endogenous tomato genes—forming the capsaicin pathway to yield spicy tomatoes.

Aims

- Identify necessary chili pepper genes to complement endogenous tomato genes through comparative expression analysis.
- Clone these genes from chili pepper for tomato transformation.
- Overexpress these genes in tomato fruit and leaves.
- Evaluate the presence of capsaicin in transformed tissue.

Identification of non-expressed genes of the capsaicin pathway in tomato

Publically available transcriptome sequences (RNA-seq) from the NCBI short reads archive database for tomato fruit, tomato leaf, chili pepper fruit and leaf were downloaded. All genes of the capsaicin pathway were detected and expressed in the chili pepper fruit transcriptome data sets (Supplemental Figure 1). Genes within the tomato genome with at least 95% amino acid similarity were extracted from the tomato genome (Supplemental Table 1). Expression levels for these tomato genes were evaluated from leaf tissue and fruit (Supplemental Figures 2 and 3). As capsaicin only accumulates in chili pepper fruit, we also evaluated the expression levels for the entire pathway in chili pepper leaves (Supplemental Figure 4). It was expected that at least one component would be lacking in chili pepper leaves. Based on these results, only three genes of the capsaicin pathway were lacking in tomato fruit: Capsaicin Synthase (known as CS, AT3d or Pun1), Ketoacyl-ACP synthase IIIb (KasIIIb), and Putative Aminotransferase (pAMT). Interestingly, these same genes are lacking expression in tomato leaves. Again, three genes had little or no expression in chili pepper leaf: CS, KasIIIb and Acyl-CoA synthetase 1 (ACS1). Based on this bioinformatic analysis, expression of three genes, CS, KasIIIb and pAMT in tomato fruit should complete the capsaicin pathway. Additionally, Kim *et al*., 2014 conducted comparative

transcriptome analysis between tomato and chili pepper placental tissue and suggested that only three capsaicin pathway genes were lacking expression in tomato: CS, KasI (an ortholog of KasIIIb), and Branched-chain amino acid aminotransferase (BCAT). The second to last step on the phenylpropanoid side of the pathway, involves the conversion of feruloyl-CoA (a precursor of lignin biosynthesis) into vanillin. This reaction in chili pepper is theoretically catalysed by Hydroxycinnamoyl-CoA Hydratase/Lyase (HCHL), however to our knowledge, HCHL has never been identified nor characterised in chili pepper. Thus, we are unsure if this gene exists or is expressed in tomato. To bypass this problem, we cloned Vanillin Synthase (*VpVAN*) from the vanilla orchid, *Vanilla planifolia*, which catalyses a nearly identical reaction. Additionally, we cloned the enzyme Branched-chain α-Ketoacid decarboxylase E1α (BCKDH) which would complete a synthetic pathway to form capsaicin from ferulic acid and valine, both compounds with abundance in tomato fruit. When this data is taken in whole, eight genes are needed to form the capsaicin pathway in tomato (Table 1).

Gene Isolation

Seven chili pepper genes: *BCAT*, *BCKDH*, *Kas*, *KasIIIb*, *CS*, *ACS1*, and *pAMT*, were cloned into Golden Gate level-0 vectors. All genes were isolated from a from chili pepper (*Capsicum annuum* var. Cayenne) placental tissue cDNA library and domesticated to remove BpiI and BsaI restriction enzyme sites by PCR, with the exception of *ACS1* which was commercially synthesised in a domesticated form, as it contained numerous BpiI and BsaI sites. An eighth gene, Vanillin Synthase (*VpVAN*), was cloned and domesticated from a *V. planifolia* pod cDNA library. The *V. planifolia* pod was gratefully donated by the University of Cambridge Botanic Gardens. All level-0 constructs were sequenced to ensure they were correctly cloned. Thus, with these eight genes, the capsaicin pathway could be artificially produced in tomato which forms a pathway. The coding sequence for each gene were all driven by the 35s promoter from the cauliflower mosaic virus (CaMV) in level-1 Golden Gate constructs. Multiple genes were then assembled into level-2 Golden Gate constructs.

Transformation

Expression binary vectors containing the level-2 constructs were transformed into *Agrobacterium tumefaciens* and infiltrated into tomato and tobacco leaf and fruit tissue. Due to unforeseen difficulties with transient gene expression in tomato fruit, we were unable to complete transient assays. As a consequence, we tested gene expression in *N. benthamiana* (Tobacco). The largest construct infiltrated into *N. benthamiana* thus far contained four genes: *CS*, *KasI*, *BCAT* and *VpVAN* which was designed to confirm the hypothesis by Kim *et al*. 2014 that these genes are sufficient to produce spicy tomatoes. Other lines expressed two genes: *CS* and *VpVAN*, or one gene: *VpVAN*. To confirm all genes were co-expressing, RNA from the *N. benthamiana* leaves was isolated and converted to cDNA and subjected to quantitative PCR (qPCR). All genes expressed higher than a housekeeping gene control (*PP2A*). These plants were then screened for accumulation of capsaicin.

Capsaicin detection

Extraction and estimation of capsaicinoid content followed the spectroscopy methods of Gonzalez-Zamora *et al*., 2015. Briefly, *N. benthamiana* infiltrated leaves were harvested at five and seven days post infiltration, dried in an oven at 65^{o}C for 48 hours and ground to a uniform powder. Each sample was boiled in HPLC grade Acetonitrile for 90 minutes at 80^{o}C to extract capsaicin, filtered through a 0.45µm pore and were measured on a spectrophotometer between 215nm and 300nm. Capsaicin absorbs UV light at 280nm. Four replicates were used per gene construct. All the *N.benthamiana* samples absorbed light at 280nm (A_{280}), including a non-transformed *N. benthamiana* control. Lines that expressed Capsaicin Synthase (CS), the terminal enzyme in the pathway, had higher A_{280} levels as compared to the non-infiltrated *N. benthamiana* (Figure 2). It is possible that these higher A_{280} values in the *N. benthamiana* lines that are expressing CS mean that they are accumulating capsaicin. This result needs to be confirmed with additional analytical methods.

Future work and conclusion

Larger gene constructs with up to the complete set of eight genes remain to be tested *in N. benthamiana*. Once we can confirm capsaicin accumulation in *N. benthamiana*, we will transform tomato with the same gene construct. Additional analytical methods will be used to verify the presence of capsaicin in transformed samples.

Thus far, it is promising that transformation of only a few chili pepper genes is capable of producing capsaicin in Solanaceae. The results of this work hopefully help to improve the understanding of capsaicin evolution in Solanaceae. This would serve as a good model to produce secondary metabolite pathways in plants.

Table/Figure Captions

Table 1. Summary of candidate capsaicin pathway genes to generate spicy tomatoes. All these genes are expressed highly in chili pepper fruit during capsaicin production.

Figure 1. Engineering the capsaicin pathway in tomato. Genes in red were cloned to complete the pathway in tomato. All genes originate from chili pepper with the exception of *VpVAN* which was isolated from the vanilla orchid (*Vanilla planifolia*), as the chili pepper enzyme for this step is not yet known.

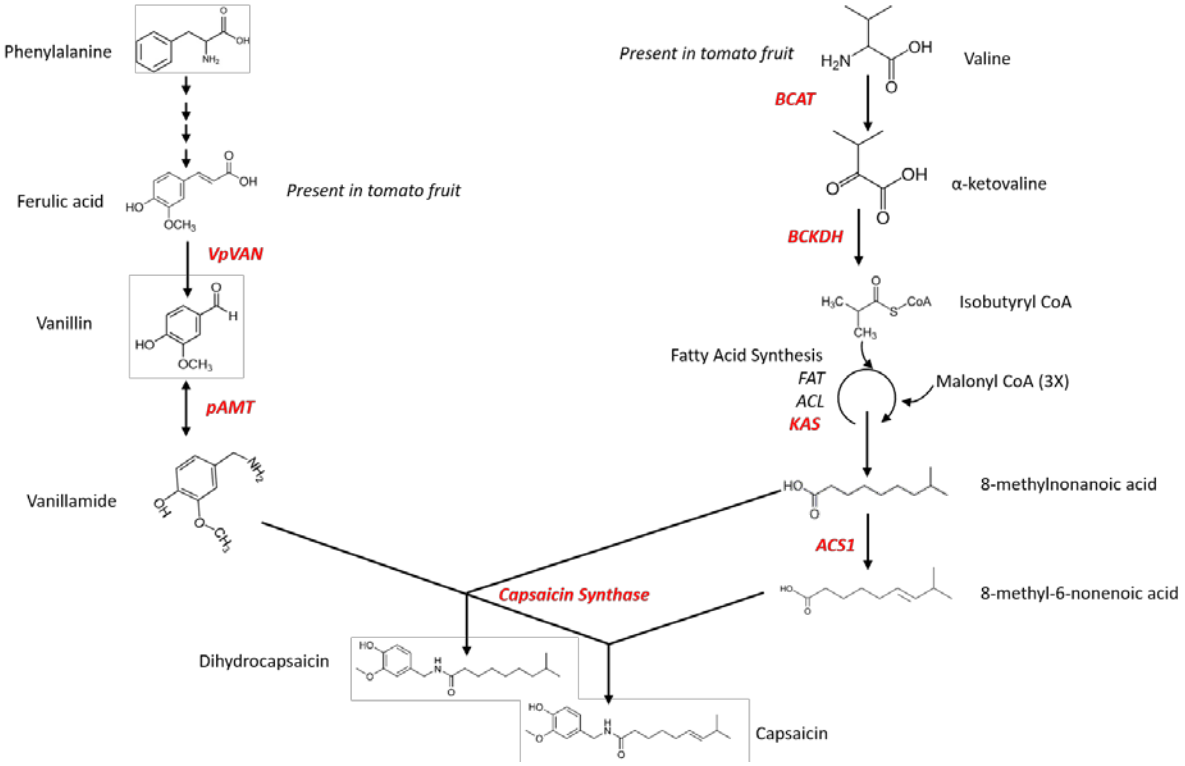
Figure 2. Spectrophotometric measurements of *N. benthamiana* lines infiltrated with capsaicin pathway genes to test for the accumulation of capsaicin. A white arrow indicates the absorbance wavelength for capsaicin (280nm). The top (dark blue) line represents the spectrum of chili pepper placental extract (positive for capsaicin), the bottom (light brown) line represents non-infiltrated *N. benthamiana* leaf (negative for capsaicin), the remaining lines are infiltrated *N. benthamiana* leaves that expressed genes of the capsaicin pathway which are indicated by the key at 5 days post infiltration (5d) or 7 days post infiltration (7d). Gene expression was confirmed by qPCR.

Expenditure:

- Gene cloning/synthesis of capsaicin pathway enzymes—Approximately £1600
- Transient expression of genes in *N. benthamiana* leaves and tomato fruit—Approximately £1150
- Capsaicin quantification costs — Approximately £100.
- Travel and sample shipping costs between Cambridge and Norwich — Approximately £250

Follow on plans:

We plan to continue experiments. So far we have been successful in expressing four genes of the capsaicin pathway in *N. benthamiana*. We currently are continuing infiltration of several more constructs which include a greater number of genes until we reach expression of all eight genes in *N. benthamiana*. Preliminary capsaicin screening via spectroscopic measurements indicates that our infiltrated lines may be producing capsaicin. We plan to further test these lines with more powerful analytic chemistry approaches (Liquid or Gas Chromatography – Mass Spectroscopy, LC/GC-MS) to confirm this result. GC-MS is a definitive means of quantifying the abundance of a compound and its molecular formula which will verify that we are measuring capsaicin. The additional £1000 will be useful to cover more transformation and quantification costs. Should this be successful, we aim to proceed with stable tomato transformation to recover a line able to produce capsaicin.



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