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Fruit specific variability in capsaicinoid accumulation and transcription of structural and regulatory genes in *Capsicum* fruit

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Abstract

Accumulation of capsaicinoids in the placental tissue of ripening chile (*Capsicum* spp.) fruit follows the coordinated expression of multiple biosynthetic enzymes producing the substrates for capsaicin synthase. Transcription factors are likely agents to regulate expression of these biosynthetic genes. Placental RNAs from habanero fruit (*C. chinense*) were screened for expression of candidate transcription factors; with two candidate genes identified, both in the ERF family of transcription factors. Characterization of these transcription factors, *Erf* and *Jerf*, in nine chile cultivars with distinct capsaicinoid contents demonstrated a correlation of expression with pungency. Amino acid variants were observed in both ERF and JERF from different chile cultivars; none of these changes involved the DNA binding domains. Little to no transcription of *Erf* was detected in non-pungent *C. annuum* or *C. chinense* mutants. This correlation was characterized at an individual fruit level in a set of jalapeño (*C. annuum*) lines again with distinct and variable capsaicinoid contents. Both *Erf* and *Jerf* are expressed early in fruit development, 16–20 days post-anthesis, at times prior to the accumulation of capsaicinoids in the placental tissues. These data support the hypothesis that these two members of the complex ERF family participate in regulation of the pungency phenotype in chile.

Keywords

Capsicum; fruit pungency; capsaicinoids; transcription factors

1. Introduction

Chile peppers (*Capsicum* spp.) are among the most important vegetables and spice crops grown worldwide. The fruit is particularly valued as a source of vitamins and of pungency, a

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Supplementary Information

Fig. S1. Habanero placental ERF is a unique member of *Erf* family and contains motifs of tomato ERF2.

Fig. S2. Habanero placental JERF is a unique member of *Erf* family and contains motifs of tomato JERF1/ERF73.

Fig. S3. Multiple sequence alignment of JERFs from nine chile cultivars

Fig. S4. Multiple sequence alignment of ERFs from nine chile cultivars

Fig. S5. Phylogram of ERF/JERF proteins from *C. annuum*, *C. chinense* and *S. lycopersicum*.

trait unique to this crop plant [1]. With only a few exceptions, all of the species in the genus synthesize capsaicinoids, the pungent compounds in their fruit [2, 3]. Capsaicin and dihydrocapsaicin are the two most abundant capsaicinoids; these odorless and colorless compounds are synthesized in the epidermis of the placenta and are stored in vesicles on the surface of this tissue [4].

Pungency in *Capsicum* is both a qualitative trait and a quantitative trait. The degree of heat, or the concentration of capsaicinoids that accumulate is inherited quantitatively [5, 6], while the ability to be hot or pungent is inherited simply, controlled by the dominant gene *Pun1* (also called *C*) on chromosome 2 [7]. A candidate gene for *Pun1* was identified as *At3*, an acyltransferase, which maps to the same location as *Pun1* on chromosome 2 [8, 9]. Three alleles for *At3* in non-pungent varieties have been identified; *pun1*¹, in *C. annuum*, has a deletion of the promoter and the first exon [8]; *pun1*², in *C. chinense*, has a smaller deletion in the first exon, also resulting in an inactive gene [9]; and *pun1*³, in *C. frutescens* has a deletion in the carboxy terminal of the second exon [10]. A second locus for non-pungency was found by Votava and Bosland in *C. chinense* missing the vesicles on the placental, *Loss of Vesicle (Lov)* [11]; the chromosomal map location of this mutation is not known.

The capsaicinoid biosynthetic pathway is complex and requires intermediates from two distinct pathways (Fig. 1) (reviewed in [1, 12]). Phenylalanine is the precursor for vanillylamine, synthesized in part by the phenylpropanoid pathway; and leucine or valine are the precursors for the synthesis of a branched chain fatty acid, eg. 8-methyl nonenoic acid, synthesized by the fatty acid synthase complex. Finally, capsaicinoid synthase combines the vanillylamine and fatty acid chain and to make capsaicin [13]. In an earlier work, we demonstrated that the transcripts for many of the enzymes on both branches of the capsaicinoid pathway share similar patterns of accumulation; transcripts are more abundant early in fruit development and more abundant in pungent *Capsicum* varieties [9, 14, 15]. These results suggest an important role for transcription factors in the coordination and regulation of the expression of pungency in *Capsicum*. In this current study we used four biosynthetic genes to monitor transcription activity of these biosynthetic pathways: *Pal* on the phenylpropanoid pathway [15], *Kas* and *FatA* on the FAS pathway [14], and *At3* a candidate for capsaicinoid synthase [8, 9].

The key role transcription factors play in regulation of secondary metabolites has been known for decades as some of the early mutants in anthocyanin pigment accumulation were the result of mutations in transcription factors [16]. The roles of transcription factors in regulation of plant secondary metabolic pathways have been reviewed [17–19]. Members of many of the classic transcription factor families have been associated with roles in secondary metabolism: MYB [20, 21], bZIP [22], bHLH and WD40 [17] and AP2/ERF [23]. Transcription factor ESTs are regularly detected in screens of transcriptomes for genes associated with secondary metabolite accumulations in Solanaceae fruits [24–26]. The expression profiles of candidate transcription factors in placental samples from pungent and non-pungent *Capsicum* fruit though have not been investigated in detail. Transcription levels of two bZIP transcription factors were included in a panel of genes but full details in support of the annotation of these genes as transcription factors was not provided [8]. More recently, transcriptome analyses identifying differentially expressed transcripts in comparisons of pericarp and placenta of pungent *Capsicum* fruit have also detected transcription factors among a number of candidate genes for the capsaicinoid pathway [27].

The current study was conducted to identify and characterize candidate genes for transcription factors likely to play a role in coordinating the transcription of the structural genes on the capsaicinoid pathway. The screening strategy selected cDNA clones with DNA sequence similarity to motifs found in known transcription factors. Transcription factor

genes expressed in a placental-specific pattern and with increased expression in fruit with increased pungency were identified. Further, as we were aware that there is a great deal of fruit to fruit variability in expression of the pungency phenotype [28, 29] we developed methods for fruit specific characterization of expression of these genes and direct confirmation of fruit specific capsaicinoid levels.

2. Materials and methods

2.1 Plant Material

Plants were grown from seed in Metro-mix 360, in a greenhouse on the New Mexico State University campus in Las Cruces, NM USA. Plants were irrigated twice a day with a drip system and fertilized with Osmocote (14-14-14), every 2–3 months. *Capsicum* germplasm included in this study are listed in Table 1. Organs and tissues collected for RNA isolation were immediately dissected and frozen in liquid nitrogen. For the time course studies, all fruit on the plants were tagged at anthesis. Two months after the first fruits were tagged all the fruit were harvested from plants at the same time. Placentas were removed and cut into ~1 cm pieces and were placed into conical tubes with 4 mL 2-propanol for 30 s with mixing. The 2-propanol was decanted and saved for capsaicinoid determinations and the placentas were frozen in liquid nitrogen for RNA extraction.

2.2 Nucleic acid isolation, cloning and DNA sequencing

Recombinant phage or plasmids were generated and characterized as described earlier [15]. DNA sequences of clones were analyzed with BLASTX (<http://www.ncbi.nlm.nih.gov/BLAST/>) to predict their function based on similarity to other known gene products. Tissues were stored at –80 °C prior to RNA isolation by an acid phenol and chloroform as described [15]. For RNA isolation from individual jalapeño fruits, the Qiagen RNeasy Plant Mini Kit was used. Methods for DNA sequencing, northern blot analysis, and PCR amplification followed standard protocols as described earlier [30, 31].

2.3 Polymerase Chain Reaction Methods

RT-PCR libraries from the placental RNA (3 µg) of varieties were made by reverse transcribing RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Amplicons were cloned and sequenced using standard protocols [30]. For each gene from each cultivar/variety, six to seven clones were sequenced; the primers used for PCR are listed in Table 2. All DNA sequence files were aligned using DNASTAR software.

The level of expression of candidate transcription factors and pathway genes were determined by quantitative reverse transcriptase PCR (qRT-PCR) analysis as described earlier [31]. Primers used for qRT-PCR are listed in Table 3. Standard curves were made using 10-fold dilutions with known concentration of each gene template to determine the reaction optimization of each template. Absolute quantification of each transcript was interpolated from the standard curves.

2.4 Pungency analysis

Capsaicin and dihydrocapsaicin were quantified using fluorescence detection following HPLC separation as described earlier [32]. Total capsaicinoids (capsaicin and dihydrocapsaicin concentrations summed) were reported.

2.5 Protein Sequence Analysis

Multiple sequence alignments of predicted ERF and JERF amino acid sequences were performed using METALIN [33] at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?

[page=npsa_multalin.html](http://npsa_multalin.html). A phylogram of 34 tomato and chile ERF and JERF sequences was generated from a multiple sequence alignment created using Clustal Ω, ver. 1.2.0 (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The predicted amino acid sequence for the ERF from *C. chinense* was submitted to the homology modeling application at SWISS-MODEL. This application identified a template for structural modeling based on amino acid sequence similarity and then generated secondary structure predictions and tertiary structure predictions [34–36]. The quality of the tertiary structure was evaluated by QMEAN4 [37].

3. Results

3.1 Selection of candidate transcription factor genes

A set of eighteen cDNA clones predicted to encode transcription factor genes were identified in two cDNA libraries already available: *C. annuum* CM334 root tissues [38] and *C. chinense* habanero placenta tissues [15]. The DNA sequences of these clones predicted gene products that had high similarity scores with motifs found in transcription factors. These clones were used as probes to determine if any of these classes of transcription factors were expressed in the target fruit organs. Hybridization to the fruit transcripts by these clones was expected due to the conserved domains shared by family members of the transcription factor gene family. Northern blot analysis was performed to screen these clones to detect genes with higher expression in fruit than other organs and higher expression in fruit from pungent varieties than non-pungent varieties. Blots with RNA from flower, leaf, root, fruit wall, as well as placental samples from immature and maturing placenta from both pungent and non-pungent chile lines were hybridized with probes of each of the eighteen different candidate transcription factors. The majority of the clones hybridized to transcripts in multiple organs and were equally expressed in placental tissues of pungent and non-pungent chiles. However, two clones, (GenBank IDs: CA847557 and DV643197) detected transcripts that were more abundant in placental tissue than other organs and more abundant in pungent placental tissue than in placental tissue from non-pungent varieties.

The clone CA847557 is predicted to encode a *Jerf*, *Jasmonate and ethylene responsive factor*. Transcripts for this gene accumulated to higher levels only in mature (green/orange) placental tissue of pungent habanero fruit and much lower in flower and immature (green) placental tissue of pungent habanero fruit. In leaf, fruit wall, root and placental tissue of non-pungent habanero, no transcripts were detected. The clone DV643197 is predicted to encode an *Erf*, *ethylene-responsive factor-like protein*. On northern blots, transcripts for this gene were only detected in fruit wall and immature (green) placental tissue of pungent habanero while no transcripts were detected in any other tissues. These *Erf* and *Jerf* clones were identified in the *C. annuum* root cDNA library. We screened the *C. chinense* placental cDNA library with these clones to obtain the ortholog/paralogs for *Erf* and *Jerf* expressed in the target organ, habanero placenta.

3.2 Isolation of Erf and Jerf candidate transcription factors from habanero placental cDNA library

A placental cDNA from *Erf* was cloned from *C. chinense*, the clone contained a 1074 nucleotide sequence with a 795 nucleotide ORF encoding 265 amino acids with 119 nucleotide 5'-UTR and 152 nucleotide 3'-UTR. The *Erf* cDNA from *C. chinense* had greater than 90% amino acid identity to the gene from *C. annuum* CM334 root cDNA library. However, the 5' UTR and the 3' UTR sequences of the *Erf* from *C. annuum* CM334 root cDNA library and from *C. chinense* placenta cDNA library were significantly different. These sequences are deposited in GenBank as KF060657 and KF060658. The *C. chinense* gene was 98% identical to both the DNA sequence and the protein sequence of the *Erf* characterized in *C. annuum* by Lee et al [39]. A multiple sequence alignment of these

protein sequences are provided in Fig. S1. Based on these alignments, both the CM334 root clone and the habanero placenta clone were full length.

The placental *C. chinense* clone for *Jerf* was a 1190 partial nucleotide sequence with a 1052 nucleotide ORF encoding 350 amino acids and 138 nucleotide 3'-UTR. Aligning this sequence using blast demonstrated that the cDNA clone from the placenta library was not full length and was missing all of the 5'UTR and ~78 nucleotide encoding the first 26 amino acids. The DNA and predicted amino acid sequences for this placental *Jerf* clone were 99% identical to a putative ethylene-responsive element binding protein (*Jerf1*) from GenBank (DQ412079). The *Jerf* clone from the CM334 root library and the *Jerf* clone from the *C. chinense* placenta library had only 50% amino acid identity. These sequences are deposited in GenBank as KF169943 and KF169944. A multiple sequence alignment of the habanero placental JERF and related JERFs and ERFs from tomato and pepper are provided in Fig. S2.

3.3 Allelic variability in ERF and JERF in chile varieties with different pungency levels

To test the hypothesis that unique variants of transcription factors are associated with differences in pungency or capsaicinoid accumulation, we compared the predicted amino acid sequences of ERF and JERF in nine different cultivars with different degrees of pungency (Table 1). Primers were designed to produce amplicons encompassing most of the coding region of the gene (Table 2).

Among the nine different chile varieties, only one amino acid polymorphism was detected in the JERF sequences, A240T. The alanine containing version of this protein was found in Bhut Jolokia, Tabasco and non-pungent habanero, all the other varieties had threonine at this position. All of the cultivars in *C. annum* and habanero, had threonine at position 240 (Fig. S2, Fig. S3). In contrast, nine different polymorphisms were detected in the predicted ERF protein sequences in these varieties (Fig. 2A). The amino acid sequence of habanero was set as the reference sequence, alignments of the ERF sequences in all nine cultivars is presented in Fig. S4. There were no amino acid differences in the sequence of ERF in three hottest cultivars, Bhut Jolokia, habanero and Tabasco. However, the amino acid sequence of ERF in all of the non-pungent cultivars had the same amino acid substitutions in three positions, N149D, I199T, and L240V. There were also amino acid substitutions that were found in only one or two cultivars, for example T20S and A65G were detected in Early Jalapeño and Canary.

Of the three amino acid changes associated with pungency level, N149D, I199T, and L240V, two substitutions could be expected to have effects on the protein structure or function. Asparagine is a neutral amino acid while aspartic acid is a charged amino acid (N149D), and isoleucine is an aliphatic hydrophobic amino acid while threonine is a polar hydrophilic amino acid (I199T). These amino acid changes would reduce the hydrophobic character of the protein in the regions of positions 149 and 199 in the ERF expressed in the no-pungent fruit. These changes could alter the ability of the variant ERF to bind to other proteins.

None of the cultivar specific amino acid polymorphisms in ERF, however appear to impact the DNA binding domain, depicted in green font in Fig. 2B. The secondary and tertiary structures of this region in the chile ERF (amino acids 75 to 131) was modeled using the structure solved for this domain in an ERF from *Arabidopsis thaliana* [40], 1gcc (Fig. 2C, 2D). The QMEAN4 score for the chile protein fit to the model was 0.77, indicative of a reasonable prediction.

3.4 Expression analysis of candidate transcription factors and four pathway genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The transcript expression profiles of four enzymes on the capsaicinoid pathway were determined using qRT-PCR (Fig. 3A–D). Very low transcript levels for *Kas*, *FatA* and *At3* were detected in the non-placental samples, fruit wall, flower, leaf and root. In contrast, *Pal* transcripts were abundant in these non-placental samples. Maximal transcript levels for each of these four genes was detected in immature placental tissues of pungent fruit, habanero in the case of *Pal*, Bhut Jolokia in the case of *Kas* and Early Jalapeño in the case of *FatA* and *At3*. Transcripts for these genes were also more abundant in immature placenta tissue than placenta from turning (green/orange) or mature (fully orange) fruit.

Transcript accumulation was then quantified for two candidate transcription factors, *Erf* and *Jerf* (Fig. 3E–F). Again there were varietal differences in transcript accumulation, *Jerf* transcript accumulation was slightly higher among the most pungent varieties, but there was transcription of this gene in all chile varieties. In contrast, transcripts of *Erf* accumulated to relatively higher levels in pungent varieties, Bhut Jolokia, habanero and Tabasco compared to mild and non-pungent varieties. Transcription of *Erf* and *Jerf* was slightly higher in immature placenta compared to other organs. During fruit development, transcript levels decreased for *Erf* in placental samples.

3.5 Variability in capsaicinoid level in fruit from an individual plant

Transcript levels for capsaicinoid pathway genes are generally correlated with fruit pungency levels as indicated in Fig. 3. These comparisons are made across a range of cultivar types, representing different species and different fruit shapes and sizes. To eliminate these sources of variability in a comparison of fruit pungency with transcript levels, we first characterized the capsaicinoid content in individual fruit collected at specific developmental stages, days post-anthesis (DPA) in six jalapeño lines, PX205, PX206, PX207, PX211, PX212 and P105, bred to produce pungency levels ranging from 0 to 60,000 Scoville heat units (Table 1). While we collected preliminary data on all six lines, we report here the detailed results from only three lines: PX207, PX211 and P105 (Fig. 4); the data from the other three lines demonstrated the same trends.

No capsaicinoids were detected in the non-pungent line P105 (Fig. 4C) at any time during fruit development. On average the fruit in PX207 contained more capsaicinoid than fruit from PX211, and capsaicinoid content increased in fruit that were older, 20 DPA vs 50 DPA. However, there were dramatically different levels of capsaicinoid in different fruits of the same age; in PX211 fruit that were 45 DPA, ranged in capsaicinoid content from 0 to 450 ppm (Fig. 4B). The same was true for fruit from PX207, at 45 DPA fruit had capsaicinoid content from 100 to 1400 ppm (Fig. 4A). While the fruit-to-fruit variation was large, trend-lines indicated that higher capsaicinoid abundance tended to occur in older fruit with the highest content observed in fruit 40 to 60 DPA.

3.6 Expression analysis of four pathway genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Transcript accumulation levels for four capsaicinoid pathway genes were determined in individual fruits from jalapeño varieties bred for different pungency levels, PX207, PX211 and P105. Different fruits with different ages for PX207 from 16 to 41 DPA, for PX211 from 19 to 42 DPA, and one fruit at 40 DPA from P105 were selected. The transcripts quantified by absolute qRT-PCR for *Pal*, *Kas*, *At3* and *FatA* are presented in Fig. 5A–D. The fruit within each variety are organized by increasing maturity (DPA). The capsaicinoid content of each of these fruit is presented in Fig. 5G.

Transcript levels for *Pal*, *Kas*, *FatA* and *At3* were usually higher in younger fruit compared to the older fruit (Fig. 5A–D). This developmental pattern was observed in fruit from both PX207 and PX211, and transcript levels for these four genes were usually highest in PX207 compared to PX211 or P105. Among fruit from PX207, only one fruit at 18 DPA did not fit this pattern for transcript levels. As expected, the capsaicinoid levels in these fruit were quite variable (Fig. 5G). Some fruit at immature stages had higher levels of capsaicinoids than fruit at later DPA.

3.7 Expression analysis of two transcription factor genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The RNA samples isolated from the fruit characterized for capsaicinoid levels and pathway gene transcripts were also used for analysis of transcription factor transcript abundance by qRT-PCR (Fig. 5E–F). Transcripts for *Erf* were abundant in young fruit and dropped to very low levels after 30 DPA in PX207 and PX211 (Fig. 5E). *Erf* transcripts were more abundant in young PX207 fruit than young PX211 fruit. Transcripts for *Jerf* were also more abundant in young fruit but accumulation persisted in PX211 for longer time points, 35 DAP (Fig. 5F). In the more pungent line, PX207, a similar pattern was observed with higher levels of *Jerf* transcripts in younger fruit.

4. Discussion

Transcription factors with differential expression in chile fruit, specifically placental tissue, were identified using the extensive phenotypic variability in *Capsicum* for fruit pungency. We hypothesized that regulatory transcription factors for capsaicinoid accumulation would be transcribed at higher levels earlier in fruit development, at higher levels in placenta than other organs, and at higher levels in pungent fruit compared to mild fruit. Using this approach we identified two transcription factors with differential patterns of RNA expression that fit our model. These patterns of expression were observed in the transcription factors, *Erf* and *Jerf* isolated as cDNA clones from habanero placental tissue. Detailed characterization of these differential patterns of expression were performed in jalapeño lines with distinct pungency levels.

Erf best fit our model for a regulatory transcription factor since transcripts for this gene were more abundant in placenta early in fruit development in both pungent lines of jalapeño, and less abundant later in fruit maturity. *Erf* transcripts were higher in placental samples from the more pungent line PX207, intermediate expression in PX211 fruit, the intermediate pungency line and low expression in the non-pungent line, P105.

This study also reports the fruit-level variability in transcription coupled with fruit level capsaicinoid chemical analyses. Our results of fruit level variability in capsaicinoid level with these jalapeño lines expand the results in other *Capsicum* fruit, *C. annuum* and *C. frutescens* [28, 29]. The jalapeño lines characterized here also showed high fruit to fruit variability for capsaicinoid content. The average capsaicinoid content of fruit from the higher pungency in lines was higher than the average for milder lines, and as fruit matured the capsaicinoid content increased although there was a great deal of variability among these fruit.

In earlier work [41], we identified several sequence motifs present in the promoter regions of selected capsaicinoid biosynthetic genes. One of these “CCTTAGA” was found in the promoters of *Acl*, *FatA* and *Ca4H* in multiple chile cultivars. This DNA sequence element is also found in a gene in *Catharanthus roseus* also believed to be regulated by a JERF (Y10182) [42]. While we did not characterize expression of *Acl* or *Ca4H* in this study, their

transcription patterns are similar to that of *FatA*, i.e., increased in placental tissue and more abundant in pungent placenta [14, 15].

While some plant secondary metabolites are induced in response to pathogens or abiotic stresses, many others are developmentally regulated. The synthesis and accumulation of these small molecules requires the coordinated expression of multiple enzymes, and a likely source of at least one level of regulation is the transcription of mRNA for these enzymes [17–19]. Capsaicin biosynthesis is unique to members of the *Capsicum* genus and is limited to the epidermal cells of the placenta. There may be as many as 54 biosynthetic steps required to convert primary metabolic precursors into capsaicin [12]. To date no detailed characterization of genes for transcription factors associated with this pathway have been reported. The placental specific and pungency enhanced expression patterns for *Erf* and *Jerf* reported here are consistent with a role in regulation of pungency in chile.

Tomato and chile, are both cultivated crops in the Solanaceae family. While many aspects of the phenotypes of these plants are unique, their genomes as might be expected have extensive syntenic regions [43]. Further, there are numerous examples of orthologous genes important in the regulation of many aspects of tomato and chile fruit development [44]. *Capsicum* fruit, unlike tomato, are considered non-climacteric and are not expected to synthesize or respond to ethylene [26]. However as we demonstrate here, two transcription factors known to be responsive to ethylene in tomato, appear to play a role in chile fruit development. Perhaps the role of the *Erf* and *Jerf* transcription factors in chile does not require activation by an ethylene receptor but occurs following some other signal as has been proposed by others [26, 45]. Jasmonic acid will increase the concentration of capsaicinoid precursors in *Capsicum* cell cultures and in the presence of salicylic acid increase the conversion of these precursors into capsaicin [46–48]. A direct effect by jasmonic acid on transcription of capsaicinoid pathway genes though was not tested.

The *Erf* family is large and complex, in *Arabidopsis thaliana*, 122 members have been identified [49]. In tomato, Sharma et al [50] identified 85 unique *Erfs* that resolved into 11 clades based on protein sequence alignments with the tomato and Arabidopsis ERFs. In tomato, the *Erf* family includes the smaller in number *Jerf* gene family, SLERF73 is identical to the tomato JERF1. Gene specific expression of specific *Erfs* has been detected in tomato developmental stages with particular emphasis on fruit maturation [24, 51] and in response to biotic or abiotic stresses [50, 52–54]. The term ethylene in the name of this gene family “Ethylene Response Factor” may be misleading, as not all members of the family are responsive to ethylene. Inclusion of a gene in the family is based simply on the presence of the AP2/ERF domain, not on a demonstrated response to the hormone ethylene. In fact several members of the gene family in tomato are not responsive to ethylene [55]. These authors prefer to organize the *Erf* family based on patterns of expression rather than the clades based on protein sequence similarity. They have further demonstrated that members of this family have distinct patterns of expression and distinct affinities for promoter elements [55]; not all members of this large gene family bind to the canonical GCC box. There are two examples of *Erf* from *Capsicum*, both identified based on expression in response to biotic and abiotic stresses, CaPF1 [56] and CaERFLP1 [39]. These two members of the *Erf* family are distinct and most similar in amino acid sequence to the JERF identified in this study from habanero placental tissue (Fig. S3).

Osorio et al. [26] identified a transcript in ripening *C. annuum* fruit that was increased at 51–57 DPA and was annotated in their study as ERF3. Using a transcriptome approach they also monitored transcripts associated with ethylene production or response and did not see any change in these transcripts during *C. annuum* fruit ripening. Lee et al. [45] monitored transcription factor gene expression in ripening pepper using a microarray approach and

identified distinct patterns of expression of transcription factors including genes annotated as AP2/EREB. In both of these recent studies, the transcripts in the ripening fruit were from the pericarp and the increase in transcription of the ERF classes of transcription factors was late in fruit development during color formation. The ERF and JERF transcription factors described in our study are expressed in a different fruit tissue, the placenta, and are increased at much earlier times in fruit development. These results suggest that multiple members of the *Erf* family are used in specific fruit tissues to regulate fruit ripening, including perhaps the accumulation of transcripts for capsaicinoid biosynthetic genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Identification of transcription factors with placental expression patterns.
- Amino acid polymorphisms in ERF correlate with pungency phenotype.
- Fruit to fruit variability in capsaicin quantified in jalapeño lines.
- *Erf* mRNA levels reflect heritable pungency in *Capsicum* spp.

Fig. 1.

Schema of capsaicinoid biosynthetic pathway. Phenylalanine is the precursor for vanillylamine synthesized by the phenylpropanoid pathway; valine is a precursor for the branched chain fatty acids (eg. 8-methyl-6-nonenoyl-CoA) synthesized by the branched chain fatty acyl synthase (FAS) pathway.

Fig. 2. ERF polymorphisms and predicted structure of DNA binding domain. **A.** Predicted amino acid polymorphisms in *Erf* transcripts in placental RNA sequences from nine varieties differing in pungency levels (dark grey rows, very pungent; light grey rows, mildly pungent; unshaded rows, non-pungent (NP)). Variant amino acids relative to the habanero sequence are shown in bold and underline font. **B.** Amino acid sequence of ERFs from three chile varieties, conserved DNA binding domain indicated in underlined green font, predicted polymorphisms relative to habanero sequence in red font. **C.** Predicted secondary structure

of DNA binding domain in chile ERF modeled on 1GCC. **D.** Predicted tertiary structure of DNA binding domain in chile ERF modeled on 1GCC.

Fig. 3. Gene expression in chile varieties and organs measured by qRT-PCR of **A.** *Pal*; **B.** *Kas*; **C.** *FatA*; **D.** *At3*; **E.** *Erf*; **F.** *Jerf*. Total RNA from chile varieties: non-pungent jalapeño (Ca nJa), non-pungent habanero (Cc nHa); mild New Mexico 6-4 (Ca 64); pungent varieties: jalapeño, (Ca Ja), tabasco (Cf Ta), habanero (Cc Ha), Bhut Jolokia (Cc BJ). Grey bars indicate immature placental tissues from varieties, or habanero placental tissue from turning (G/O) or mature (O) fruit. RNA was also collected from habanero flower (Fl), root (Ro), fruit wall (Fw) and leaf (Lf), indicated by open bars.

Fig. 4. Scatter plots comparing total capsaicinoid per individual fruit versus fruit age (days post anthesis) for three jalapeño lines: **A.** PX207 (n=129); **B.** PX211 (N= 102); **C.** P105 (n=51).

Fig. 5. Gene expression in jalapeño lines differing in pungency measured by qRT-PCR analysis of **A. *Pal***; **B. *Kas***; **C. *FatA***; **D. *At3***; **E. *Erf***; **F. *Jerf*** and **G. fruit specific capsaicinoid levels**. Placental RNA from single fruits of three jalapeño lines PX207 (dark grey bars), PX211 (light grey bars) and P105 (open bars) were assayed in triplicate for transcript levels as indicated. Capsaicinoid levels for these fruit were measured prior to RNA extraction. Age of fruit in days post anthesis is indicated.

Table 1

Capsicum cultivars characterized in this study.

Species	Cultivar	Capsaicinoid SHU ^a	ERF, JERF variability	Fruit specific capsaicinoids	qRT-PCR
<i>C. annuum</i>	Canary	0	X		
	np Jalapeño	0	X		X
	NuMex Joe E. Parker	1,200	X		
	New Mexico 6-4	1,200	X		X
	Early Jalapeño	8,000	X		X
	Jalapeño P105	0		X	X
	Jalapeño PX212	2,000		X	
	Jalapeño PX211	9,000		X	X
	Jalapeño PX205	25,000		X	
	Jalapeño PX206	40,000		X	
	Jalapeño PX207	60,000		X	X
<i>C. chinense</i>	np Habanero PI 1721	0	X		X
	Habanero PI 1720	300,000	X		X
	Bhut Jolokia	1,000,000	X		X
<i>C. frutescens</i>	Tabasco	40,000	X		X

^a SHU, Scoville Heat Units

Table 2Primer pairs for amplification of coding region of *Erf* and *Jerf*.

Gene	primers	Amplicon (bp)
<i>Erf</i>	fwd 5' -GTGTGGTGGAGCAATTCTTG-3' rev 5' -AGCTCCATCAAGCCACCA-3'	741
<i>Jerf</i>	fwd 5' -GCTGACTTTCAGGACTTCAAGG-3' rev 5' -GAAGGTCCAGAGATCCATCG-3'	837

Table 3

Primer pairs for qRT-PCR quantification of transcript abundances.

Gene (GenBank ID)	Primer sequences
<i>Erf</i> (KF060657)	fwd 5' -TATTTTTCGGTTTAGGAGAATGG-3' rev 5' -TTTGAAGACAAAATAGGGCAATG-3'
<i>Jerf</i> (KF169944)	fwd 5' -TAAGCCAACACGCACCTTC-3' rev 5' -GTTTGGAGACAACATAACTG-3'
<i>Pal</i> (AF081215)	fwd 5' -CAACAGCAACATCACCCCATGTTTGC-3' rev 5' -GCTGCAACTCGAAAAATCCACCAC-3'
<i>Kas</i> (AF085148)	fwd 5' -GTGTACAAATGCCAGCAAGCTCTG-3' rev 5' -GATTCCACTTTGTCCCTCGAGAAG-3'
<i>FatA</i> (AF318288)	fwd 5' -CAATGTTGTCTCGGGGAGTTTTTC-3' rev 5' -CTCTCTCTCTCATTAGTAGCTACAGC-3'
<i>At3</i> (AY819027)	fwd 5' -CCTCATGCATCTCTTGCAGAGAGCATAG-3' rev 5' -GTCGTATGATCACGAGTAACGCTAGACC-3'