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In Silico Screening of the S_H3 Locus in the *Coffea canephora* and *Coffea arabica* Genomes to Identify Candidate Resistance Genes Against Coffee Leaf Rust (CLR)

Marc Lenard T. Merlin[1] and Ma. Carmen A. Ablan-Lagman[1,2,*]
[1] *Department of Biology, College of Science, De La Salle University*
[2] *Practical Genomics Laboratory, Center for Natural Sciences and Environmental Research, De La Salle University*
**Corresponding Author: ma.carmen.lagman@dlsu.edu.ph*

Abstract: Resistance (R) genes encode for proteins that function to confer resistance against invading pathogens. In coffee (*Coffea spp.*), the complex, multi-gene S_H3 locus was reported to provide robust resistance against *Hemileia vastatrix,* the causative agent of coffee leaf rust (CLR). In this study, an *in silico* screening of the S_H3 locus in *C. canephora* and *C. arabica* genomes was performed. Six of the ten gathered molecular markers of the S_H3 locus, namely BA-48-21O-f, Sat244, Sat160, SP-M5-SH3, Sat281, and BA-42-21B-r, gathered from literature indicated that the locus is located in chromosome 3 of both coffee genomes, spanning the region between 3-16 Mbp. Possible resistance genes were obtained from the Ensembl Plants and Coffee Genome Hub databases, and were further filtered using the Orange program to determine which are associated with defense response using their Gene Ontology (GO) term names/keywords. In *C. canephora*, there were 94 possible resistance genes, which gave 65 protein hits according to Protein BLAST. There were 82 and 65 genes in the *C. canephora* and *C. eugenioides* subgenomes of *C. arabica*, which gave 33 and 30 protein hits according to Translated BLAST results. Functional characterizations of the proteins in the context of defense responses were elucidated from UniProt-KB and Interpro as well as published literature. Maps were constructed using the chromoMap app to visualize the locations of the genes in the chromosome.

Key Words: Coffee; Leaf rust; S_H3 resistance locus

1. INTRODUCTION

Coffee pests and diseases are regarded as one of the major reasons for the decrease in volume of coffee production both in the Philippines and in the world (DA-BAR, 2022). Coffee leaf rust (CLR) is considered a devastating disease in coffee, causing tremendous yield losses ranging from 15-80% (Waller et al., 2007) and financial losses of over USD 1 billion annually (Wellman, 2021 in Koutouleas & Collinge, 2022). Its causative agent, the basidiomycete *Hemileia vastatrix*, is an obligate biotrophic parasite (Talhinhas et al., 2017). The disease is characterized by the presence of yellow to orange infective urediniospores, on the underside (abaxial) of infected leaves (Krishnan, 2017).

Documentation on the occurrence of CLR in the Philippines is scarce. Although it has been recorded in the list of pathogens by the Department of Agriculture (DA-ATI, 2007), there are limited updated national reports and local publications regarding its status. However, more recently, a loop-mediated isothermal amplification (LAMP)-based method for the determination of resistant and susceptible coffee varieties has been developed by researchers from University of the Philippines-Diliman (Sembrano & Odejar, 2024). Further, in this era of artificial intelligence, deep convolutional models (DCM) (Montalbo & Hernandez, 2020) and image processing technology workflow (Carpio et al., 2019) were developed to identify CLR-infected. These current

technologies that aim to control CLR suggest that the disease is of agricultural and economic significance for the country.

Before a pathogen completely succeeds in inflicting a disease, it undergoes several plant defense mechanisms, mainly classified into two—passive and active defense mechanisms. Passive defense mechanisms are pre-formed physical and chemical barriers (Silva et al., 2022). However, certain pathogens have adapted to and evolved mechanisms to overcome these passive defenses. In this arms race, plants have developed a second line of defense at the cellular and molecular levels, the proteins encoded by resistance (R) genes, which constitute the active defense mechanisms (Takken & Joosten, 2000). These proteins are responsible for detecting the pathogen-associated molecular patterns (PAMPs) and effectors so that defense responses can be mounted against the invading pathogens (Silva et al., 2022).

Coffee resistance to rust is mainly conferred by R genes called S_H (susceptibility to *Hemileia*) genes, nine of which are discovered (S_H1-9) while others are not characterized yet (S_H?), occurring either singly or in combination (Sera et al., 2022; Talhinhas et al., 2017). The S_H3 gene locus derived from *Coffea liberica* (Alkimim et al., 2016) confers durable resistance to CLR which, according to tandem array studies, is a complex multi-gene cluster (Ribas et al., 2011). To date, it has been the only rust resistance gene that is genetically and physically characterized in coffee (Silva et al., 2022). In addition, several studies have concentrated in identifying molecular markers associated with the S_H3 gene locus. The utilization of this gene in coffee breeding programs is especially important in developing rust-resistant cultivars (Sera et al., 2022).

Highlighting its importance, this study conducted an *in silico* screening of possible resistance genes located in the multi-gene cluster S_H3 gene locus both in the *C. canephora* and *C. arabica* genomes. Further, the identity of the proteins encoded by the possible resistance genes were elucidated, shedding light on the mechanism by how they contribute to the defense response during rust infection. The locations of these genes were also visualized through the construction of chromosome maps.

2. METHODOLOGY

2.1. Determining the Location of the S_H3 Locus

Molecular markers of the S_H3 gene locus, together with their primers, were gathered from published literature. The reference genomes of *C. canephora* (name: AUK_PRJEB4211_v1; accession: GCA_900059795.1; accessed on 14 July 2023) and *C. arabica* (name: Cara_1.0; accession: GCA_003713225.1; accessed on 14 July 2023) were accessed in the National Center for Biotechnology Information (NCBI) GenBank database. The binding sites of the molecular markers were determined by performing Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/ accessed on 14 July 2023) (Ye et al., 2012). The Primer-BLAST results were then subjected to Nucleotide BLAST (blastn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 17 July 2023) to identify corresponding sequences in the *C. canephora*, *C. arabica*, and *C. eugenioides* genomes. The chromosomal region analyzed was delimited if it satisfies any of the following criteria stated by Nagaño et al. (2022): (1) whose product size is close (within 100 bp) to the expected size as published in literature; (2) primer mismatches are few; (3) have corresponding binding sites in other coffee genomes as determined by blastn; and (4) must not have several binding sites. Additional base pairs upstream and downstream the markers were made as allowance to the delimited region of interest (Nagaño et al., 2022).

2.2. Gathering Candidate Genes in the S_H3 Locus of C. canephora and C. arabica

Protein-encoding genes within the S_H3 gene locus of *C. canephora* were obtained from Ensembl Plants (https://plants.ensembl.org/index.html accessed on 18 July 2023) (Kinsella et al., 2011), whereas those of *C. arabica* were from the Coffee Genome Hub (https://coffee-genome-hub.southgreen.fr/ accessed on 18 July 2023) (Denoeud et al., 2014). Data relevant to the genes, including the gene name/gene stable ID, location (gene start and end), protein stable ID (if available), and Gene Ontology (GO) terms/keywords, were downloaded in Comma Separated Values (CSV) format).

The candidate genes obtained were further screened if they are probably involved in defense response during CLR infection through filtration of their GO terms/keywords using Orange program (https://orangedatamining.com/) (Demsar et al., 2013). The GO terms/keywords included those stated by Nagaño et al. (2022) with modifications, including "defense response," "defense response to fungus," "defense response to virus," "abscisic acid-activated signaling pathway," "ethylene-activated signaling pathway," "flavonoid biosynthetic process," "gene silencing by RNA," "negative regulation of defense

response to virus," "negative regulation of phosphoprotein phosphatase activity," "plant-type hypersensitive response," "response to abscisic acid," "response to biotic stimulus," "response to ethylene," "response to strigolactone," response to virus," "virus-induced gene silencing," and "systemic acquired resistance." The filtered data were exported in CSV format.

2.3. Construction and Visualization of Genes

Input files were first constructed using Microsoft Excel and saved as tab-delimited format (TAB): (1) a chromosome file containing the chromosome name with their location (gene start and end coordinates) and (2) an annotation file containing the gene name, chromosome name, and location (gene start and end coordinates). Chromosome maps were constructed using the chromoMap app version 0.4.1 (https://lakshay-anand.github.io/chromoMap/index.html accessed on 26 July 2023) (Anand & Rodriguez-Lopez, 2022). Running the chromoMap app required another program called Docker (https://docs.docker.com/get-docker/ accessed on 26 July 2023). Parameters such as the color and size of the maps were modified, and then downloaded in HTML format and subsequently converted in Portable Network Graphics (PNG) format).

2.4. Identification of Proteins Encoded by Genes in the S_H3 Locus

Proteins encoded by filtered candidate genes in *C. canephora* were identified using Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins accessed on 25 July 2023). Since protein stable ID are not available for *C. arabica* genes obtained from the Coffee Genome Hub, proteins were identified through Translated BLAST (blastx) (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 25 July 2023). The functions of the proteins obtained were searched in UniProt-KB (https://www.uniprot.org/ accessed on 19 July 2023) and in published literature.

3. RESULTS AND DISCUSSION

3.1. The Location of the S_H3 Gene Locus

Ten molecular markers (Table 1) of the S_H3 resistance locus were gathered from the works of Nagaño et al. (2022) and Yu et al. (2021). Three of them were simple sequence repeats (SSRs) or microsatellites and seven were sequence characterized amplified region (SCARs).

Primer BLAST revealed that only eight of the ten molecular markers bound to the *C. canephora* genome either have both the forward and reverse primers bound (BA-48-21O-f, BA-124-12K-f, SP-M8-SH3, SP-M5-SH3, and Sat 281) or have unique binding sites (Sat244, Sat160, and BA-42-21B-r). On the other hand, only seven of the ten molecular markers bound to the *C. arabica* genome, either having both the forward and reverse primers bound (BA-48-21O-f, SP-M16-SH3, BA-42-21-B-r, Sat281, Sat160) or having unique binding sites (SP-M8-SH3, SP-M5-SH3).

Table 1. Molecular markers of the S_H3 resistance locus. F-forward primer; R-reverse primer.

1	**BA-48-21O-f** (SCAR)	F: ACAGTGAATTCCCCAAGCAC R: ACTTGGCAGGCGTAATTGAA
2	**BA-124-12K-f** (SCAR)	F: TGATTTCGCTTGTTGTCGAG R: TGCAGATTGATGGCACGTTA
3	**Sat244** (SSR)	F: GCATGTGCTTTTTGATGTCGT R: GCATACTAAGGAAATTATCTGA CTGCT
4	**SP-M8-SH3** (SCAR)	F: GAATTCAGCGACGATTG R: GATTTGGTGGAAGGGAGC
5	**Sat160** (SSR)	F: TGCTTAGGCACTTGATATAGGA R: CACGTGCAAGTCACATACTTTA
6	**SP-M5-SH3** (SCAR)	F: TTCACGATCCAAGAAGCA R: AGCATGCATTGTAGAAAAA
7	**Sat281** (SSR)	F: TCTTCGTCTTTGCTATTGGT R: TATTAACGTCCATCCACACA
8	**BA-42-21B-r** (SCAR)	F: CACACACAGCCTAAGCATCAA R: GGATTGACTCGACTCACCAA
9	**SP-M16-SH3** (SCAR)	F: TTAACTGGAAACTTGGCTTG R: ATCTAGCTTTGGAACATCGT
10	**SP-M18-SH3** (SCAR)	F: CTATTTGGTGTGGGAAGTAAC R: CTACATCCACGGAGAGAAAC

Of the eight molecular markers that bound in the *C. canephora* genome, only seven indicated correspondences in the *C. arabica* and/or *C. eugenioides*

genomes, as revealed by Nucleotide BLAST. BA-48-21O-f has unique correspondences in both *C. arabica* and *C. eugenioides*. The markers BA-124-12K-f, Sat244, and SP-M8-SH3 have correspondences only in *C. eugenioides*, whereas Sat160 and SP-M5-SH3 have correspondences in *C. arabica* genome only. BA-42-21B-r have several correspondences in both genomes, while Sat 281 does not have any corresponding sites.

Of the seven molecular markers that are bound in the *C. arabica* genome, only three have correspondences in the *C. arabica/C. eugenioides* genomes. The marker BA-48-21O-f have unique binding sites in both *C. arabica* and *C. eugenioides*. Sat 160 has a correspondence only in *C. canephora*, whereas BA-42-21B-r have correspondences in both genomes. Similar to above, Sat281 does not have any corresponding sites.

With the results of the Primer BLAST and Nucleotide BLAST, the region to be analyzed can now be delimited. For *C. canephora*, seven markers satisfied the criteria to be included in the analysis, namely BA-48-21O-f, Sat244, SP-M8-SH3, Sat160, SP-M5-SH3, Sat281, BA-42-21B-r. The binding sites of all of these markers are located in chromosome 3, and indicate that the probable location of the S_H3 resistance locus in *C. canephora* spans from 3-16 Mbp.

For *C. arabica*, only three markers satisfied the criteria to be included in the analysis, namely BA-48-21O-f, Sat160, and BA-42-21B-r. Similarly, the binding sites of these markers are located in chromosome 3 of the *C. canephora* and *C. eugenioides* subgenomes in *C. arabica*, and marks the 3-16 Mbp as the probable location of the S_H3 resistance locus.

3.2. Candidate Genes in the S_H3 Locus of C. canephora and *C. arabica*

The search for protein-encoding genes in the S_H3 resistance locus of *C. canephora* in the Ensembl Plants database displayed 747 unique genes. Upon filtering of the GO terms relevant to defense response to CLR infection using the Orange program, the list was narrowed down to 94 genes (Supplementary Data 1). Of this, 62 genes noted the GO term "defense response," whereas 25 genes noted the term "defense response to fungus." The abundance of these defense response-related genes is an indication of the general resistance of *C. canephora* and its derivatives against fungal pathogens.

For *C. arabica*, the search for genes in the Coffee Genome Hub database gave 1,206 genes for the *C. canephora* subgenome (3c), which was then narrowed down to 82 genes (Supplementary Data 2). Of these, the GO terms "defense response" and "abscisic acid-activated signaling pathway" frequently occurred, with 12 and 10 genes, respectively. On the other hand, there were 1,056 genes in the *C. eugenioides* subgenome (3e), which was filtered down into 65 genes (Supplementary Data 3). Similar to 3c, the most frequently occurring GO terms are "defense response" and "abscisic acid-activated signaling," with 9 and 6 genes, respectively. The Coffee Genome Hub database indicated keywords for genes which are not GO names. The most frequently occurring are genes that encode for proteins with a CC-NBS-LRR domain. This protein domain has been associated with the structure of R genes (Takken & Joosten, 2000). In particular, the LRR (leucine-rich repeat) motif is a predictor of where the protein will be localized, whether intercellular or extracellular, and therefore most likely function as a receptor (Takken & Joosten, 2000) where effectors secreted by pathogens will bind (Andersen et al., 2018). The NBS (nucleotide binding site) domain functions in signaling through activation of kinases or G proteins in order to induce defense responses such as the hypersensitive response (HR) and production of reactive oxygen species (ROS) (Takken & Joosten, 2000)

Compared to the genes in the *C. canephora* genome, there are fewer defense response-related genes obtained in 3c of *C. arabica*. One potential reason for this is the incompleteness of the data available in the Coffee Genome Hub database. In fact, the annotation of *C, arabica* genes is a challenging task due to the tetraploid nature of its genome. An alternative reason is that there may be comparatively fewer genes in *C. arabica* related to defense response, offering an explanation why *C. arabica* cultivars are more susceptible not only to CLR but to other pathogens as well.

3.3. Chromosome Maps Indicating the Locations of the Genes in the S_H3 Locus

The chromosome maps constructed using the chromoMap app showing the locations of the genes in the S_H3 resistance locus in the *C. canephora* (blue) and *C. arabica* (3c-orange; 3e-violet) genomes are illustrated in Figure 1.

3.4. Proteins Encoded by Genes in the S_H3 Locus and their Functional Characterization

The Protein BLAST performed on 94 genes in *C. canephora* gave out 65 protein hits. Translated BLAST

conducted on 82 genes in the *C. canephora* subgenome (3c) gave 33 protein hits, whereas the 65 genes in *C. eugenioides* gave 30 protein hits.

The proteins encoded by genes found in the S_H3 resistance locus were loosely classified into eight groups: (1) proteins involved in post-transcription and translation; (2) proteins in hormone signaling (receptors and transcription factors); (3) enzymes in secondary metabolite biosynthesis; (4) major allergen Pru ar 1-like (pathogenesis-related proteins); (5) argonaute protein; (6) putative late blight resistance proteins; (7) protein kinases; and (8) putative disease resistance proteins containing protein domains characteristic of R genes.

Protein hits involved in post-transcription and translation were characterized to be an mRNA cap-binding protein and the subunit H of eukaryotic translation initiation factor 3 (eIF3). Cellular mRNA translation is initiated by a cap-dependent mechanism, which is attributed to the proteins that comprise the 7-methylguanosine cap located at the 5'-end of mRNA (Ramanathan et al., 2016). eIF3 is involved in the cap-dependent scanning mode of translation initiation that ultimately leads to the recognition of the start codon by the small ribosomal subunit (Kim et al., 2007). Thus, these proteins are thought to regulate post-transcription and translation processes that give rise to proteins involved in downstream defense responses.

Receptors and transcription factors for hormone signaling were also noted in the protein hits. In particular, protein hits share homology to ethylene receptor and ethylene-responsive transcription factor. The complex interaction of ethylene with other plant hormones salicylic acid and jasmonic acid has been classically related to plant defense (Silva et al., 2022). A different protein hit, the low-temperature-induced 65 kDA protein, is a protein produced in response to the hormone abscisic acid. Typically associated with biotic stress, abscisic acid in this context may imply its role in mediating plant defense response.

Secondary metabolites are compounds with various biological functions in plants. Some of the genes were found to encode for the enzyme chalcone synthase, which produces chalcone, an intermediate in the flavonoid biosynthetic pathway; and chalcone-flavonone isomerase, which catalyzes the intramolecular cyclization of bicyclic chalcones into tricyclic (S)-flavanones. Flavonoids participate in the defense response as scavengers of ROS generated by stress conditions (Panche et al., 2016), which in this case may be attributed to fungal infection. In addition, a gene was found to encode for the enzyme S-norcoclaurine synthase, which catalyzes the biosynthesis of S-norcoclaurine, a precursor to benzylisoquinoline alkaloids (Lee & Facchini, 2010). Generally, alkaloids act as signal molecules when a plant is under stress.

Several of the protein hits are major allergen Pru ar 1-like proteins (from apricot, *Prunus armeniaca*). Protein databases UniProt-KB and Interpro report that these proteins are members of the Bet v I type allergens (from birch, *Betula verrucosa*). While considered an allergen, in the context of plant defense, these proteins share homology with PR-10 (pathogenesis-related) proteins. The Bet v I family of allergens, where the Pru ar 1-like protein belongs, includes a subset of defense-related genes that are transcriptionally activated when pathogens are present (Swoboda et al., 1994).

Interestingly, protein-argonaute 10-like was noted in the protein hits. Argonaute proteins are involved in RNA interference (RNAi), which is an RNA-mediated gene silencing mechanism. In a bidirectional phenomenon called cross-kingdom RNAi, gene silencing is induced between two unrelated species of different kingdoms, which in this case coffee and the rust fungus. The short non-coding RNA and argonaute protein of the host may translocate into the fungal cells and induce gene silencing to disrupt funga;l pathogenicity genes (Mapuranga et al., 2023).

The putative late blight resistance protein confers the resistance of wild and cultivated potatoes against the potato late blight fungus, *Phytophthora infestans*. It participates in defense by triggering the hypersensitive response, thereby restricting the growth of the pathogen and controlling its spread.

Several of the protein hits are protein kinases, which generally function in the phosphorylation of molecules–a process essential in signaling pathways. These kinases contain the LRR motif, which as mentioned, predicts the intercellular or extracellular localization of the protein (Takken & Joosten, 2000).

Lastly, putative disease resistance proteins were among the protein hits. They were reported to perform roles in defense responses, such as pathogen recognition (Cooley et al., 2000), induction of hypersensitive response, and positive or negative regulation of the salicylic acid signaling pathway (Liu et al., 2020).

4. CONCLUSIONS

This study conducted an *in silico* screening of the possible resistance genes located in the S_H3 resistance locus in *C. canephora* and *C. arabica* genomes. The binding sites of the markers for S_H3 locus indicate that this region is located in the chromosome 3

of both coffee species. Mining of genes of *C. canephora* in the Ensembl Plants Database provided 94 genes in this locus, which encoded for 65 different proteins. Further, genes of *C. arabica* obtained from the Coffee Genome Hub provided 82 and 65 possible resistance genes in its *C. canephora* (3c) and *C. eugenioides* (3e) subgenomes, which encoded for 33 and 30 different proteins, respectively. The proteins were loosely classified into eight groups, each involved in a variety of responses ranging from post-transcription and translation, hormone signaling, secondary metabolite biosynthesis, signal transduction pathways, to gene silencing through RNAi. Maps were also constructed to visualize the locations of these genes in the chromosome.

The findings of this study expands the current knowledge on coffee resistance against rust. This can serve as a reference of which genes can be used in DNA barcoding aimed to discriminate between resistant and susceptible coffee cultivars, especially in the local coffee industry; as well as aid in the marker-assisted selection and development of resistant coffee cultivars. Finally, these can all lead to the establishment of an integrated pest-disease management program in the country, thus limiting the use chemical methods of control that pose threats to the health of the environment and humans, and reducing the financial burden of coffee farmers brought about by the cost of pesticides and the technology to apply it. It offers a sustainable solution to uphold the coffee industry and allow the sector to continuously thrive amid climate change.

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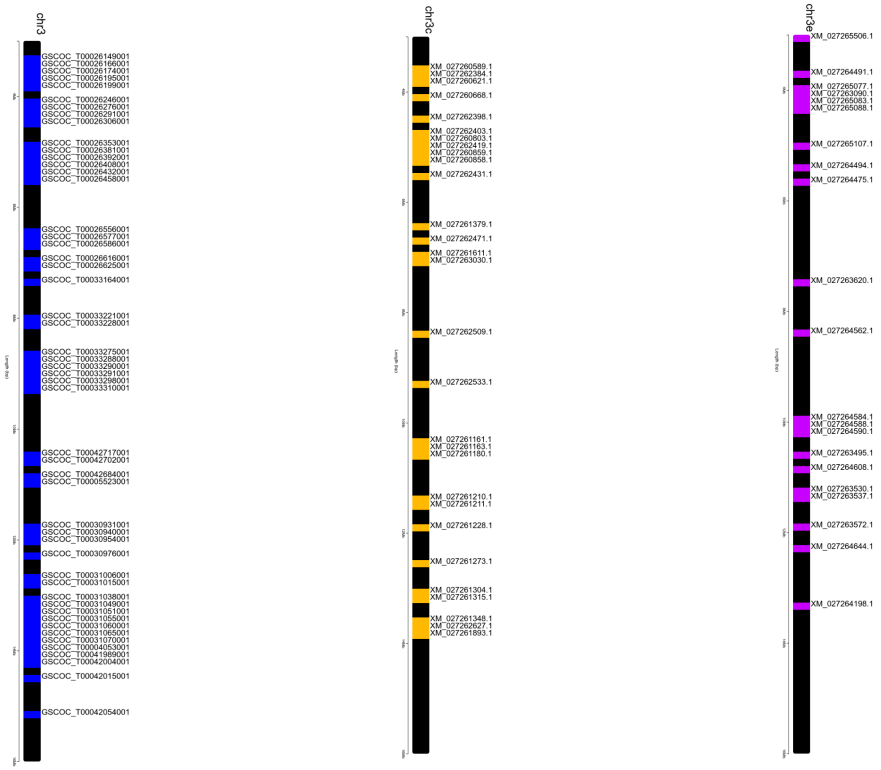

Figure 1. Chromosome maps of the S_H3 gene locus in *C. canephora* (blue) and *C. arabica* (3c-orange and 3e-violet).

6. SUPPLEMENTARY DATA

The list of genes with relevant information about them, obtained from the *C. canephora* and *C. arabica* genomes can be accessed below:

C. canephora genome:
https://bit.ly/Coffea_canephora_SH3Genes

C. canephora subgenome of *C. arabica* (3c):
https://bit.ly/Coffea_arabica_3c_SH3Genes

C. eugenioides subgenome of *C. arabica* (3e):
https://bit.ly/Coffea_arabica_3e_SH3Genes

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