A new eIF4E1 allele characterized by RNAseq data mining is associated with resistance to potato virus Y in tomato albeit with a low durability

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Received 12 July 2016 Accepted 16 September 2016

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Allele mining on susceptibility factors offers opportunities to find new sources of resistance among crop wild relatives for breeding purposes. As a proof of concept, we used available RNAseq data to investigate polymorphisms among the four tomato genes encoding translation initiation factors [eIF4E1 and eIF4E2, eIFiso4E and the related gene new cap-binding protein (nCBP)] to look for new potential resistance alleles to potyviruses. By analysing polymorphism among RNAseq data obtained for 20 tomato accessions, 10 belonging to the cultivated type Solanum lycopersicum and 10 belonging to the closest related wild species Solanum pimpinellifolium, we isolated one new eIF4E1 allele, in the S. pimpinellifolium LA0411 accession, which encodes a potential new resistance allele, mainly due to a polymorphism associated with an amino acid change within eIF4E1 region II. We confirmed that this new allele, pot12, is indeed associated with resistance to potato virus Y, although with a restricted resistance spectrum and a very low durability potential. This suggests that mutations occurring in eIF4E region II only may not be sufficient to provide efficient and durable resistance in plants. However, our study emphasizes the opportunity brought by RNAseq data to mine for new resistance alleles. Moreover, this approach could be extended to seek for putative new resistance alleles by screening for variant forms of susceptibility genes encoding plant host proteins known to interact with viral proteins.

INTRODUCTION

One of the main challenges of plant breeders is to identify new sources of resistance to pathogens. Crop wild relatives are the main source of such resistances, which can be monitored through the direct phenotyping of collections of accessions with the pathogen of interest. However, this approach is both time-consuming and costly. Allele mining, which entails looking for polymorphism within genes of interest, represents a complementary approach to plant phenotyping in order to isolate new sources of resistance to pathogens among the natural variation. Viruses are characterized by a particularly small genome and rely on host factors to infect their host (Fraser, 1990). Hence, polymorphism within those host factors can be associated with the plant resistance, a mechanism dubbed as loss of susceptibility (Pavan et al., 2010; van Schie & Takken, 2014).

Such an approach has been exemplified by the characterization of resistance to viruses based on the eukaryotic translation initiation factors eIF4E (Robaglia & Caranta, 2006; Wang & Krishnaswamy, 2012). Translation initiation factors 4E (i.e. eIF4E and the isoform eIFiso4E) are essential components of the eukaryotic cell that initiate translation by binding to the mRNA cap structure at the 5′ end of most mRNAs. They are encoded by a small multigene family (Browning & Bailey-Serres, 2015). In the last decade, those factors have been shown to be associated with resistance to a broad range of positive-sense ssRNA viruses and especially potyviruses, including potato virus Y (PVY) and tobacco etch virus (TEV) (Wang & Krishnaswamy, 2012). Natural resistance to potyviruses in lettuce (Nicaise et al., 2003),
pepper (Ruffel et al., 2002), pea (Gao et al., 2004) and tomato (Ruffel et al., 2005) has been shown to rely on non-synonymous substitutions in the eIF4E coding sequences. Those substitutions are mainly located in two regions, named I and II, located near the cap-binding pocket in the eIF4E (Robaglia & Caranta, 2006). However, the precise involvement of those two regions in resistance remains unclear. In pepper, a large set of eIF4E1 resistance alleles has been characterized (see below). While mutations in region I of pepper eIF4E1 are associated with resistance to PVY, the additional mutations in region II might be associated with enlargement of the resistance spectrum to TEV (Charron et al., 2008; Yeam et al., 2007). However, no natural allele harbouring only resistance-associated mutations in region II has been isolated so far among the pepper natural variation. Beside the need for new resistance alleles, it is also crucial to determine the minimal set of mutations within eIF4E allowing the broadest resistance spectrum possible, in the wake of new breeding technologies such as clustered regularly interspaced palindromic repeats/CRISPR associated protein 9 (CRISPR/Cas9) (Andersen et al., 2015). So far, the proof of concept of using this technology to develop resistance has been illustrated by knocking out eIF4E and eFiso4E genes in Cucumis sativus and Arabidopsis thaliana, respectively (Chandrasekaran et al., 2016; Pyott et al., 2016). However, recent work in tomato suggests that, given the redundancy effect among eIF4E genes, CRISPR/Cas9 technology should rather be used to design functional alleles by introducing non-synonymous mutations in the eIF4E coding sequence, rather than using null alleles (Gauffier et al., 2016).

Given their central role in resistance to RNA viruses, eIF4E family members have also been a target of choice for proof-of-concept studies as well as for a large number of allele mining strategies. In pepper, direct sequencing of eIF4E1 cDNAs sampled on different accessions has characterized nine pvr2 recessive resistance alleles to PVY and TEV (Charron et al., 2008; Ruffel et al., 2002). Similar approaches using the EcoTILLING strategy or high resolution melting analysis have extended this repertoire up to 22 alleles (Ibiza et al., 2010; Jeong et al., 2011). Such studies have also been carried out in other crops such as melon (Nieto et al., 2007), barley (Hofinger et al., 2011) and pea (Konečná et al., 2014), demonstrating the usefulness of the approach. In the last decade, the development of high-throughput sequencing, especially RNAseq, has provided large amounts of publicly available data and precise knowledge of the gene expression patterns in major crop plants, as well as giving access to the polymorphism associated with amino acid (aa) changes in the encoded proteins. Those data represent a largely untapped resource to mine for new alleles of interest (Barbasch et al., 2016). However, it is important to check whether the available data, often collected to provide insight on plant evolution and analysis of speciation, in tomato for example (Koenig et al., 2013; Pease et al., 2016), are well suited for the specific purpose of allele mining. Compared to the large amount of eIF4E resistance alleles discovered in Capsicum spp., only one resistance allele of the eIF4E1 tomato orthologous gene was discovered in the accession PI247087 (hereafter PI24). This allele is associated with a large resistance spectrum to several isolates of PVY and TEV (Legnani et al., 1995, 1996). Because PI24 is an accession of the wild species Solanum habrochaites, introgression of the resistance into cultivated tomato is difficult (Bernacchi & Tanksley, 1997; C. Gauffier, C. Lebaron & J.-L. Gallois, unpublished results). In this study, we investigated polymorphisms among the four tomato genes encoding translation initiation factors [eIF4E1 and eIF4E2, eIFiso4E and the related gene new cap-binding protein (nCBP)] to seek for new potential resistance alleles. To this end, we looked for polymorphism among RNAseq data obtained for 20 tomato accessions, 10 belonging to the cultivated type Solanum lycopersicum and 10 belonging to the closest related wild species Solanum pimpinellifolium (Sarah et al., 2016 and available at http://www.arcad-project.org/) and isolated one new eIF4E1 allele, pvr1, in the S. pimpinellifolium LA0411 accession. Our results show the high feasibility of using RNAseq data to mine for new resistance alleles and can be extended to other host susceptibility factors. Moreover, our data bring new insights into the minimal set of non-synonymous mutations in the eIF4E1 genes that can be useful to design CRISPR/Cas9 variant alleles with a broad and durable resistance spectrum.

RESULTS

Analysis of RNAseq data among 20 tomato accessions allows the characterization of a new eIF4E1 allele in the S. pimpinellifolium LA0411 accession

In plants, translation initiation factors are encoded by a small multigene family. In tomato, we previously identified three genes encoding initiation factors 4E, including one isoform eFiso4E and two eIF4E homologues, eIF4E1 and eIF4E2, that are involved in susceptibility to potyviruses (Piron et al., 2010; Ruffel et al., 2005). Verification of the eIF4E2 annotation on the Sol Genomics database (https://solgenomics.net/) revealed that the exon structure of the eIF4E2 gene differs from the gene model characterized earlier, as the fifth exon was not included. The annotation (Solyc02g021550) was revised accordingly. By analysing available expressed sequence tags in the GenBank database and using the reference tomato genome (Tomato Genome Consortium, 2012), we further identified a tomato gene encoding a homologue to the A. thaliana nCBP, characterized by the substitution of two of the eight tryptophan residues conserved in eIF4E proteins by phenylalanine and tyrosine, respectively (Rud et al., 1998; Fig. S1, available in the online Supplementary Material).

Recently, transcriptome data of a set of 20 tomato accessions were obtained through the ARCAD project (http://www.arcad-project.org/) using paired-end HiSeq2000 sequencing, aiming at deciphering the molecular footprints
of domestication in crop species, such as tomato or rice. The experimental design was based on a comparative approach of 10 accessions belonging to cultivated tomato *S. lycopersicum* and *S. lycopersicum* var. *cerasiforme* and 10 accessions from the closest wild species *S. pimpinellifolium* (Table S1). The cDNA sequences for the four genes encoding initiation factors, namely elf4E1, elf4E2, elfiso4E and nCBP, were retrieved (Table 1), and polymorphisms called according to the approach detailed previously (Nabholz et al., 2014) were sought.

Nucleotide sequences could be retrieved for the four genes in the 20 accessions, showing that those genes are well expressed in leaf tissues used to create the cDNA libraries. No non-synonymous aa changing substitution was found among elf4E2, elfiso4E and nCBP coding sequences. Moreover, no nucleotide polymorphism at all could be found in either elf4E2 or elfiso4E, whereas two silent substitutions were found in the nCBP coding sequence, including one common to all *S. pimpinellifolium* species sequenced. In comparison, four new haplotypes within the elf4E1 coding sequence were characterized for tomato elf4E1 and, as previously shown for the elf4E1 homologue in pepper, all polymorphisms were associated with aa changes within the elf4E1 coding sequence (Fig. 1). For haplotypes 1–3, only one mutation was found, which was located outside the regions I and II where mutations associated with resistance are usually located (Robaglia & Caranta, 2006). More significantly, the LA0411 accession (haplotype 4) displayed two non-synonymous substitutions within the elf4E1 sequence, including one mutation located at position 112 in region II, where an aspartic acid (D) is replaced by a glycine (G) (D112G, Fig. 1).

The presence of this polymorphism was confirmed in the LA0411 elf4E1 allele by Sanger sequencing. The rest of the study was focused on the LA0411 allele, as it encodes a protein with an aa change within region II, but not in region I. It was of particular interest to see whether the region II mutation by itself provides resistance to potyviruses.

**The elf4E1 allele of accession LA0411 is a new pot1<sup>2</sup> resistance allele to two PVY isolates**

We first checked whether the *S. pimpinellifolium* LA0411 accession displayed any resistance to potyviruses. Thus, LA0411 plants were mechanically inoculated with three isolates of PVY (LYE90, N605 and SON41g) and three isolates of TEV (CAA10, S103 and HAT). The resistance phenotype was assayed by titrating viral accumulation by ELISA at 21 days post-inoculation, in the systemically infected leaves. Susceptible *S. lycopersicum* plants M82, as well as the highly resistant *S. habrochaites* PI24 accession, which harbours the elf4E1–pot1 allele, were used as positive and negative controls, respectively. Our results showed that the LA0411 plants display resistance to the PVY isolates LYE90 and N605, and are susceptible to PVY SON41g as well as to all three TEV isolates tested (Table 2 and Fig. S2). Therefore, LA0411 is associated with a reduced but significant resistance spectrum to potyviruses.

The genetic basis of LA0411 resistance to potyviruses was further assessed using PVY N605 isolate. LA0411 plants were manually crossed with the susceptible *S. lycopersicum* M82 accession. F1 plants were crossed to each parent to produce resistant and susceptible backcrosses, respectively. F2 were produced by selling F1 plants. All populations were tested for resistance or susceptibility to PVY N605 (Table 3). Both the F1 and the susceptible backcross progeny plants were found to be fully susceptible to PVY N605, suggesting the presence of a recessive resistance gene. In comparison, resistant plants did segregate among the resistant backcross (BC1R) and the F2 progeny, in accordance with the presence of a single recessive gene.

Finally, an allelism test was carried out between the broad resistance allele elf4E1–pot1 which originates from the wild accession PI24 (Parrella et al., 2002) and the LA0411 elf4E1 allele. Wild species belonging to *S. habrochaites*, such as PI24, are notably difficult to cross with cultivated tomato (Bernacchi & Tanksley, 1997). Therefore, near-isogenic lines that have been generated by introgressing the elf4E1–pot1 allele into the elite Mospomorist (*S. lycopersicum*) cultivars were used, hereafter named NIL-pot1 (C. Gauffier and others, unpublished results). F1 plants issued from the cross between NIL-pot1 and LA0411 were all resistant to PVY N605 (n=20 plants), suggesting that the monogenic recessive resistance in LA0411 is allelic to elf4E1–pot1, and that the resistance in the *S. pimpinellifolium* LA0411 is caused by mutations in its elf4E1 allele. Consequently, we propose to name this allele as elf4E1–pot1<sup>2</sup>. To confirm this allelism test, F1 plants generated between NIL-pot1 and LA0411 were self-crossed and F2 plants were tested for their resistance to PVY N605. Although the F2 plants were mostly resistant to PVY N605 as expected given the allelism

<table>
<thead>
<tr>
<th>Gene</th>
<th>Identifier</th>
<th>Chromosome</th>
<th>CDS length</th>
<th>Haplotypes</th>
<th>New haplotypes with aa changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>elf4E1</td>
<td>Solyc03g0058700</td>
<td>3</td>
<td>696</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>elf4E2</td>
<td>Solyc02g021550</td>
<td>2</td>
<td>663</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>elfiso4E</td>
<td>Solyc09g090580</td>
<td>9</td>
<td>503</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>nCBP</td>
<td>Solyc10g080660</td>
<td>10</td>
<td>672</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

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Table 1. Haplotypes of the tomato translation initiation factors 4E found in the RNAseq ARCAD dataset

CDS, coding DNA sequence. The haplotypes’ references correspond to the sequences from *S. lycopersicum* M82 accession.
between eIF4E1–pot1 and eIF4E1–pot12 (38 resistant plants out of 40 plants inoculated with PVY N605), two plants (representing 5%) were found to be fully susceptible to the virus. This low occurrence of susceptibility could be caused by resistance breakdown of the eIF4E1 resistance allele. Consequently, we further investigated the durability of eIF4E1–pot12-mediated resistance to PVY N605.

Resistance associated with eIF4E1–pot12 is easily overcome by PVY N605-derived variants

Partial breakdown of the resistance harboured by the S. pimpinellifolium accession LA0411 could have been missed due to the small numbers of plants assayed. Therefore, two more resistance tests were carried out, by assaying at least 50 plants per genotype. All M82 plants were found to be susceptible, while all PI24 plants were resistant to the virus. In comparison, LA0411 plants showed a surprising high level of susceptibility ranging from 43 to 56% of the plants (Table 4).

Resistance breakdown by PVY in pepper and tomato has been associated with non-synonymous substitutions occurring within the viral VPg (viral protein genome-linked) cis-tron (Charron et al., 2008; Moury et al., 2004). Sequencing of the reverse transcription (RT)-PCR-amplified VPg cistron of the PVY N605 isolate after its propagation in three independent susceptible M82 plants did not reveal any mutation in comparison with the original inoculum. Then, 25 independent LA0411 plants infected by PVY N605 were sampled, and the corresponding VPg cistrons sequenced to identify potential mutations associated with the gain of
virulence. All sequences showed polymorphisms associated with aa changes within the VPg coding region. These mutations were a substitution of a leucine by a serine at position 115 in all of the sequenced progenies (L115S) and an additional substitution of an isoleucine by a valine at position 139 (I139V) in nine of the sequenced progenies. This result confirms that resistance-breaking variants derived from PVY N605 were detected after a first passage on LA0411 plants. Notably, the mutation affecting L115 had previously been characterized as a mutation consistently associated with resistance breaking in pepper for the related PVY N605 variant progenies propagated in LA0411 plants and the two RB isolates, respectively, harbouring L115S and L115S+I139V substitutions in the VPg, were propagated on LA0411 symptomatic plants after a first passage were back inoculated onto LA0411 plants, 93% of the plants were infected. Therefore, the second passage allowed the increase in the number of infected plants (ranging from 62 to 93%) confirming that the L115S and L115S+I139V variants were breaking the resistance associated with eIF4E1–pot12. The remaining non-infected plants (accounting for 7% of the inoculated plants) may be due to technical variation in mechanical inoculation.

Altogether, these results show that the eIF4E1–pot12 allele is associated with a very low durability to PVY N605.

**PVY N605 resistance-breaking isolates use both eIF4E1 and eIF4E2 in tomato**

We showed previously that although the natural allele eIF4E1–pot1 was associated with resistance to most PVY and TEV isolates, a null TILLING allele knockout (KO) eIF4E1 was associated with a very narrow resistance spectrum (Piron et al., 2010). We further showed that resistance could be restored, including PVY N605, by combining null mutations affecting both eIF4E1 and eIF4E2, uncovering a redundancy effect between eIF4E1 and eIF4E2 (Gauffier et al., 2016). To investigate how the resistance-breaking (RB) PVY N605 variants are able to overcome the eIF4E1–pot12-mediated resistance, we looked at the resistance status of previously characterized tomato TILLING loss-of-function mutants (Gauffier et al., 2016; Piron et al., 2010) towards those RB variants. The reference isolate PVY N605 and the two RB isolates, respectively, harbouring L115S and L115S+I139V substitutions in the VPG, were propagated on susceptible M82 plants and inoculated on a single KO mutant affecting eIF4E1 and eIF4E2 (hereafter Δ4E1 and Δ4E2 plants, respectively) and on plants combining both mutations Δ4E1 Δ4E2 (Table 6). Δ4E1 and Δ4E2 plants were susceptible to all three PVY isolates tested, showing that PVY N605 isolate and its derived eIF4E1–pot12 RB PVY

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession</th>
<th>PVY</th>
<th>TEV</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>LYE90</td>
<td>N605</td>
</tr>
<tr>
<td>S. lycopersicum</td>
<td>M82</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>S. pimpinellifolium</td>
<td>LA0411</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S. habrochaites</td>
<td>PI24</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

**Table 3.** Inheritance of resistance to PVY N605 in *S. pimpinellifolium* LA0411

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Expected ratio</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. lycopersicum M82</td>
<td>S</td>
<td>7</td>
<td>0</td>
<td>1:0</td>
</tr>
<tr>
<td>S. pimpinellifolium LA0411</td>
<td></td>
<td>0</td>
<td>7</td>
<td>0:1</td>
</tr>
<tr>
<td>(M82 × LA0411)F1</td>
<td></td>
<td>20</td>
<td>0</td>
<td>1:0</td>
</tr>
<tr>
<td>BC1S = (M82 × LA0411)F1 × M82</td>
<td></td>
<td>20</td>
<td>0</td>
<td>1:0</td>
</tr>
<tr>
<td>BC1R = (M82 × LA0411)F1 × LA0411</td>
<td></td>
<td>20</td>
<td>20</td>
<td>1:1</td>
</tr>
<tr>
<td>(M82 × LA0411)F2</td>
<td></td>
<td>26</td>
<td>14</td>
<td>3:1</td>
</tr>
</tbody>
</table>
N605 L115S and L115S+I139V variants are able to recruit either eIF4E1 or eIF4E2 to infect tomato. In comparison, plants combining mutations in both eIF4E1 and eIF4E2, D4E1/C2D4E2, in addition to being resistant to PVY N605, were also fully resistant to the RB variants PVY N605 L115S and L115S+I139V. As suggested for the pepper/PVY and pepper/TEV pathosystems, these results suggest that the mutations in the VPg of the two evolved PVY N605 isolates allow the virus to recruit the mutated form of eIF4E1–pot12 rather than hijacking new host factors in the plant. Combination of the eIF4E1–pot12 allele with a null eIF4E2 TILLING allele will be carried out to confirm this hypothesis: the resulting genotype is expected to be susceptible to the RB PVY N605 isolates.

**DISCUSSION**

In the present study, by using RNAseq data collected from 20 tomato accessions, including 10 from the wild related species *S. pimpinellifolium*, we could assess for the first time, we believe, the sequence variability among all eukaryotic translation initiation factors 4E, including the isoform eIFiso4E and the atypical related protein nCBP that had not been described yet in tomato. This new set of data is crucial in the light of the comparison with data already put forward in pepper (*Capsicum* spp.) for eIF4E1 (Cavatorta et al., 2008; Charron et al., 2008; Ibiza et al., 2010). Indeed, both *Capsicum* and *Solanum* spp., recessive resistance to these potyviruses has been characterized as relying on natural variants of eIF4E1. Finally, both plant species encode the same set of eukaryotic translation initiation factors 4E: eIF4E1, a closely related eIF4E2 protein, the eIFiso4E isoform and the nCBP (Gauffier et al., 2016; this study; J.-L. Gallois & C. Caranta, unpublished results).

Our results suggest contrasted evolution patterns within the eukaryotic translation initiation factor 4E gene family. As shown previously in pepper, the eIF4E1 gene displayed the largest number of nucleotide polymorphisms associated with aa changes. Overall, the screening of the 20 tomato accessions from the ARCAD dataset allowed us to find polymorphism within seven accessions and identify four new haplotypes coding for four distinct new eIF4E1 proteins. Those polymorphisms affected five positions within the eIF4E1 coding sequence and were all associated with aa changes. These results are in accordance with the analysis of polymorphism of the *Capsicum* eIF4E1 (Cavatorta et al., 2008; Charron et al., 2008; Ibiza et al., 2010) and suggest that, as in pepper, positive selection may act on eIF4E1 to fix new alleles. In pepper, this positive selection has been associated with co-evolution with potyviruses (Charron et al., 2008), and, consequently, a similar process could occur in tomato. In contrast with the high level of polymorphism among eIF4E1; no polymorphism was detected in eIF4E2 or eIFiso4E sequences. The results on eIFiso4E nucleotide variability in tomato differ from the relatively high diversity of polymorphism discovered among *Capsicum* spp. accessions, again associated with aa changes (Ibiza et al., 2010). Interestingly, several pepper accessions harbour a natural KO of the eIFiso4E gene, due to a deletion occurring within the gene. This loss of function, when

### Table 4. LA0411 shows lower durability to PVY N605 than PI24

Results from DAS-ELISA from two independent assays. R represents the percentage of resistant plants. n, Number of plants assayed. ELISAs were performed at 21 days post-inoculation.

<table>
<thead>
<tr>
<th>Accession</th>
<th>M82</th>
<th>PI24</th>
<th>LA0411</th>
</tr>
</thead>
<tbody>
<tr>
<td>R (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay 1</td>
<td>0</td>
<td>100</td>
<td>43</td>
</tr>
<tr>
<td>n</td>
<td>57</td>
<td>57</td>
<td>58</td>
</tr>
<tr>
<td>Assay 2</td>
<td>0</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>n</td>
<td>51</td>
<td>60</td>
<td>59</td>
</tr>
</tbody>
</table>

### Table 5. Back inoculation of PVY N605-evolved isolates on LA0411 plants is associated with a reduction of resistance

Results from DAS-ELISA. R represents the percentage of resistant plants. n, Number of plants assayed.

<table>
<thead>
<tr>
<th>Accession</th>
<th>M82</th>
<th>PI24</th>
<th>LA0411</th>
</tr>
</thead>
<tbody>
<tr>
<td>R (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVY N605</td>
<td>0</td>
<td>100</td>
<td>38</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>9</td>
<td>50</td>
</tr>
<tr>
<td>L115S</td>
<td>0</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>10</td>
<td>55</td>
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<tr>
<td>L115S+I139V</td>
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<td>100</td>
<td>7</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>9</td>
<td>57</td>
</tr>
</tbody>
</table>
combined with a elf4E1-mediated pvr2 resistance allele, is associated with resistance to several isolates of pepper vein mottle virus and chilli vein mottle virus, as well as an increase in overcoming pvr2 resistance (Hwang et al., 2009; Quenouille et al., 2016; Ruffel et al., 2006). Therefore, this nucleotide variability in the pepper elfiso4E could be driven by an involvement in susceptibility to potyviruses, and on the contrary, elfiso4E would not be involved in susceptibility to potyviruses in tomato. Indeed all elfiso4E loss-of-function tomato mutants studied so far remain susceptible to potyvirus infection (Piron et al., 2010). Finally, we report for the first time, we believe, an analysis of nucleotide diversity within the elf4E2 coding sequence. No polymorphism could be detected among the elf4E2 coding sequence for any of the 20 tomato accessions tested. elf4E2 has been shown to be a susceptibility factor for potyviruses, but only when the elf4E1 gene was knocked out, through RNAi or ethyl methanesulfonate-induced mutation (Gaufier et al., 2016; Mazier et al., 2011). More precisely, comparing natural functional elf4E1 variants and non-functional KO TILLING mutants in tomato allowed us to show that the presence of an elf4E1 natural resistance allele makes the elf4E2 factor unavailable for the potyvirus. Consequently, the stability of elf4E2 coding sequence is fully consistent with an absence of selection pressure from the viruses on elf4E2. Similar results were obtained on the pepper elf4E2 sequence variability (Charron, 2007), suggesting that a similar mechanism of redundancy between elf4E1 and elf4E2 could be found in this related species.

This study allowed us to characterize in S. pimpinellifolium accession LA0411 one new elf4E1 allele whose sequence suggests that it could provide a new resistance allele, due to the presence of an aa change within region II. Both genetic studies and resistance assays showed that this new allele is indeed a recessive elf4E1 resistance allele to PVY, hence named pot12. The elf4E1 protein encoded by this allele is characterized by two aa changes. One of these, V54K, is located at the N-terminal part of the protein, outside regions I and II where mutations associated with resistance to potyviruses are consistently located in elf4E factors in different plant species (Robaglia & Caranta, 2006). This mutation is also present on its own in two cultivated accessions from our set (LA1420 and LA0409). Both LA1420 and LA0409 plants were found to be fully susceptible to PVY N605 (10 susceptible plants out of 10) suggesting that this aa change does not play a role in resistance (data not shown). The second mutation, D112G, is located in region II, a short region predicted to locate near the cap-binding pocket in the elf4E 3D structure, and involves a substitution of an acidic residue by a non-polar one. Because it originates from a close wild relative to cultivated tomato, this allele can be easily introgressed into the cultivated tomato. However, compared with the elf4E1–pot1 resistance allele, which originates from the PL24 S. habrochaites accession and confers resistance to PVY and TEV, elf4E1–pot12 is associated with a narrow resistance spectrum as it confers PVY resistance with limited durability and no resistance to TEV. These results suggest also that efficient resistance to potyviruses in Solanaceae may require simultaneous aa changes in two distant regions of the elf4E1 protein (regions I and II); these results must be taken into account for strategies involving new breeding techniques such as CRISPR/Cas9 to engineer resistance alleles which may require two rounds of CRISPR/Cas9 gene modifications. Alternatively, one may consider combining the use of natural variation and genome modification by using the elf4E1–pot12 allele as a template to introduce mutations in region I by CRISPR/Cas9.

The development and evolution of high-throughput sequencing technologies, such as RNAseq, have a tremendous impact on plant breeding by allowing access to genome-wide genetic diversity, molecular marker development and genome-wide association studies at a reasonable cost (Barabaschi et al., 2016; Goodwin et al., 2016). Our study demonstrates also the straightforward use of RNAseq data to mine for alleles of interest, and can be implemented using new sets of data, on a larger set of accessions, that have been released (Pease et al., 2016). However, the limitations of next-generation sequencing data should be kept in mind, including the presence of false-negative single nucleotide polymorphisms (i.e. the lack of detection of actual polymorphisms), a problem already acknowledged for clinical genetic diagnostic (Huang et al., 2015; Park et al., 2015). Such false-negative rates have been assessed as being as high as 4% by comparing RNAseq data with Sanger sequences in Brassica rapa, and could account for missing potentially interesting polymorphisms (Devisetty et al., 2014). Therefore, the lack of detected polymorphism does not necessarily reflect a wild-type allele. For example, Sanger sequencing confirmed that the Cervil cultivated tomato accession, that was included in our panel, contains an elf4E1 variant (D112N) that was not detected in our RNAseq data screening.

Overall, the use of RNAseq data potentially allows the simultaneous analysis of all members from the same gene family. That is of particular interest for plant translation initiation factors 4E, given the large redundancy among the genes encoding those factors in the plant genome, as well as the specific recruitment of the different elf4E isoforms by various species of potyviruses as exemplified in A. thaliana.
(Duprat et al., 2002; Nicaise et al., 2007; Sato et al., 2005). Furthermore, such studies could be extended by investigating a large number of susceptibility candidate genes, such as the chloroplastic phosphoglycerate kinase 2 that has been shown to be associated with resistance to the potyvirus watermelon mosaic virus in A. thaliana, or the protein disulfide isomerase like 5-1, a gene involved with resistance to bmyoviruses in barley (Ouibrahim et al., 2014; Yang et al., 2014). Because of their limited genome, viruses recruit host factors to complete all the steps of their infectious cycle, and are therefore engaged in many interactions with host proteins. Several studies have allowed the characterization of plant proteins interacting with viral proteins, mainly using yeast two hybrid protein–protein interaction systems (for review see Elena & Rodrigo, 2012) or affinity purification (Martinez et al., 2016). Those host genes potentially encode susceptibility factors, and variants of those genes could possibly encode new resistance factors, mirroring the example of eIF4E proteins in potyvirus resistance. This reverse genetics approach could allow extension of the portfolio of resistance genes based on loss of susceptibility and could define potentially resistant accessions that could then be used as sources for transfer to crops.

**METHODS**

**Plant materials.** M82 (S. lycopersicum) was used as the susceptible control. The resistant accession S. habrochaites PI247087 (PI24) has been described previously (Parrella et al., 2002). LA0411 accession seeds were provided by INRA Avignon CRB (Centre de Ressources Biologiques, http://www6.paca.inra.fr/gaf). The TILLING lines KO for eIF4E1 (ΔE1) and eIF4E2 (ΔE2) were previously obtained in a S. lycopersicum M82 background (Piron et al., 2010) and the double mutant (ΔE1 ΔE2) has been previously described (Gauffier et al., 2002).

**eIF4E sequence analysis.** The RNAseq data were obtained in the framework of the ARCAD project aiming at deciphering the molecular footprints of domestication through a comparative genomic approach. Towards this objective, the experimental design relied on 10 crop and 10 wild accessions from two species (S. lycopersicum and S. pimpinellifolium) chosen on the basis of their molecular diversity. A standardized protocol was followed for each of these species in terms of RNA extraction, RNA sequencing and bioinformatic analyses as described previously (Nabholz et al., 2014). Among these species, crop tomato accessions and wild accessions were grown in the greenhouse under classical conditions. Briefly, RNA was extracted from young leaves, fruits and flowers that were pooled in a 65:20:15 proportion following their flowering and flowers that were pooled in a 65:20:15 proportion following their flowering and flowers that were pooled in a 65:20:15 proportion following their flowering. The RNAseq data were obtained in the framework of the ARCAD project aiming at deciphering the molecular footprints of domestication through a comparative genomic approach.

**Virus isolates and infection assays.** The PVY isolates LYE90v (Moury et al., 2004), N605 (Parrella et al., 2002) and SON41g (Charron et al., 2008) and the TEV isolates CAA10 (Charron et al., 2008), S103 (Ruffel et al., 2005) and HAT (Schaad et al., 2000) were propagated on Nicotiana tabacum ‘Xanthi’ before inoculating 14-day-old tomato plants. The accumulation of PVY and TEV viruses in non-inoculated upper leaves was then assayed 21 days post-inoculation by double antibody sandwich ELISA (ELISA-ELISA) using, respectively, anti-PVY (Sediag) and anti-TEV (Sediag) antisera and detection sets. Non-inoculated plants were used as healthy controls. Mean and si of absorbance values at 405 nm of samples from at least six independent plants per parental genotype and 12 independent plants per progeny were calculated. The susceptibility threshold was set as three times the mean value of healthy controls.

**ACKNOWLEDGEMENTS**

The RNAseq data production was initially supported by the Agropolis Foundation under the ARCAD project number 0900-001 (http://www.arcad-project.org/). We thank Renaud Duboscq (INRA Avignon GAF) for additional RNAseq data analysis as well as André Moretti and Carole Caranta (INRA Avignon GAF) for providing the NIL-pot1 plants. We thank the INRA Avignon Centre de Ressources Biologiques (CRB) for providing the LA0411 seeds.

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