

# Positive Darwinian Selection at Single Amino Acid Sites Conferring Plant Virus Resistance

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**Abstract** Explicit evaluation of the accuracy and power of maximum likelihood and Bayesian methods for detecting site-specific positive Darwinian selection presents a challenge because selective consequences of single amino acid changes are generally unknown. We exploited extensive molecular and functional characterization of amino acid substitutions in the plant gene *eIF4E* to evaluate the performance of these methods in detecting site-specific positive selection. We documented for the first time a molecular signature of positive selection within a recessive resistance gene in plants. We then used two statistical platforms, Phylogenetic Analysis Using Maximum Likelihood and Hypothesis Testing Using Phylogenies (HyPhy), to look for site-specific positive selection. Their relative

power and accuracy are assessed by comparing the sites they identify as being positively selected with those of resistance-determining amino acids. Our results indicate that although both methods are surprisingly accurate in their identification of resistance sites, HyPhy appears to more accurately identify biologically significant amino acids using our data set.

**Keywords** Disease resistance · *eIF4E* · Positive selection

## Introduction

The ability to detect positive selection at the molecular level has expanded rapidly as both the number of complete genome sequences and the availability of sophisticated statistical methods have increased. Maximum likelihood (ML) and Bayesian estimators have been a critical development for inferring positive Darwinian selection at the molecular level (Ford 2002; Nielsen and Yang 1998). Two commonly used statistical program packages for determining specific sites under positive selection include Phylogenetic Analysis Using Maximum Likelihood (PAML) (Yang 1997; Yang et al. 2000, 2005) and Hypothesis Testing Using Phylogenies (HyPhy) (Pond et al. 2005). These methods are attractive because they enable identification of individual codon sites under positive selection and eliminate the assumptions about population demography associated with other statistical tests of selection (McDonald and Kreitman 1991; Tajima 1989; Yang 2002). The more recently developed fixed effects likelihood (FEL) approach, applied through HyPhy, is similar but makes no previous assumptions concerning the distribution of rates across the gene. Rates are estimated independently at each site using the modified Suzuki and Gojobori method (Kosakovsky Pond and Frost 2005; Suzuki

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2004; Suzuki and Gojobori 1999). There seems to be no clear consensus regarding which of these methods is more appropriate for a given data set, but it is thought generally that PAML methods are more accurate for large data sets and prone to false-positive results with small data sets (Kosakovsky Pond and Frost 2005).

A body of literature exists that examines the power and accuracy of statistical methods for detecting positive selection (Yang 2002; Yang and Bielawski 2000). Approaches to assessing these methods generally fall into two categories: those that rely on computer simulation (Anisimova et al. 2001; Anisimova et al. 2002) and those that rely on empirical testing of genes suspected of being positively selected (Sorhannus 2003; Suzuki and Nei 2004). Both approaches are problematic. The former produces modeled results that are unconvincing without accompanying empirical data, and the latter identifies positively selected amino acids but offers no experimental evidence that significant fitness consequences exist as a result of modifications in those amino acids. Although they are often able to show which methods are more or less powerful, they provide no information regarding accuracy because they have no way of distinguishing between true- and false-positive results (Suzuki and Nei 2004).

ML and Bayesian methods are frequently used to localize the action of positive selection to specific codons and to show tight association between these sites and functionally important regions of the protein they encode (Hughes and Nei 1988; Meyers et al. 1998; Savage and Miller 2006). However, positive selection acting directly on functionally significant amino acids is rarely demonstrated because the fitness-related phenotypic consequences of individual amino acids are usually unknown (Bishop 2005). Consequently, the accuracy of site-specific tests of selection remains fundamentally in question. These criticisms compelled us to seek a system in which the fitness consequences of particular amino acids are well documented. If any sites are positively selected along such a gene, it is likely that the sites involved increase fitness.

A well-characterized plant virus-resistance gene encodes the eukaryotic translation initiation factor 4E (*eIF4E*), which functions to bind the 5' cap of messenger RNAs and recruit them to the ribosomal complex (Gingras et al. 1999). Successful potyvirus (*Potyviridae*) infection of host plants requires that the virus usurp this process: viral RNA binds directly to the *eIF4E* plant protein, a process mediated by a viral-encoded protein (VPg) linked to the 5' end of the viral RNA (Schaad et al. 2000; Shahabuddin et al. 1988). This binding ensures that viral RNA is recruited to the ribosomal complex and is translated. If this interaction is disrupted by natural mutations in *eIF4E*, the plant is resistant to infection (Leonard et al. 2000). Virus resistance has evolved independently at the *eIF4E* locus in several plant species,

suggesting that there is a strong evolutionary pressure to resist viral pathogens. This is especially remarkable given that resistance at this locus behaves recessively, and therefore natural selection can only act to preserve beneficial mutations at this site when in the homozygous recessive state. Several of the plant species known to have evolved recessive resistance genes also contain multiple alleles, each with a unique resistance spectra, as a result of coevolution with viral pathogens (Charron et al. 2008). VPg, the gene encoding the viral protein that directly binds to *eIF4E* and functions as the pathogenicity determinant, was shown to be positively selected in a previous study (Moury et al. 2004). These observations suggest that the plant species investigated have experienced strong positive selection in their evolutionary history. We hypothesize that the statistical models that identify positively selected amino acids in *eIF4E* will also accurately predict the amino acids that are critical for the resistance phenotype. Currently, a time-consuming reverse-genetics approach is the only means by which to identify the amino acids responsible for the resistance phenotype that differ among various plant species and even among alleles within the same plant species. The identification of positively selected amino acids may be an alternative approach.

In this study, we focused on *eIF4E* resistance alleles in pepper (Charron et al. 2008; Kang et al. 2005a; Ruffel et al. 2002), tomato (Ruffel et al. 2005), and pea (Gao et al. 2004). The individual amino acid changes that define resistant and susceptible *eIF4E* alleles have been carefully detailed genetically (Kang et al. 2005a, b; Ruffel et al. 2002), biochemically (Kang et al. 2005a; Yeam et al. 2007), and by functional complementation in plants (Gao et al. 2004; Ruffel et al. 2002, 2005; Yeam et al. 2007). This precise knowledge of the effects on plant survival because of single substitutions in *eIF4E* provides an a priori expectation, thus allowing us to assess the accuracy of two popular methods for inferring positive selection. Both PAML Model 8 and HyPhy FEL were compared for their ability to identify positively selected amino acids that colocalize with those directly involved in providing resistance against viral pathogens. We documented for the first time that positive selection is acting on a recessive resistance gene. We found that both methods are able to detect resistance-determining sites accurately but that HyPhy FEL does so with greater precision and power.

## Methods

### Sequence Data and Tree Construction

The *eIF4E* cDNA data set used in this study was compiled from previously published sequences from pepper

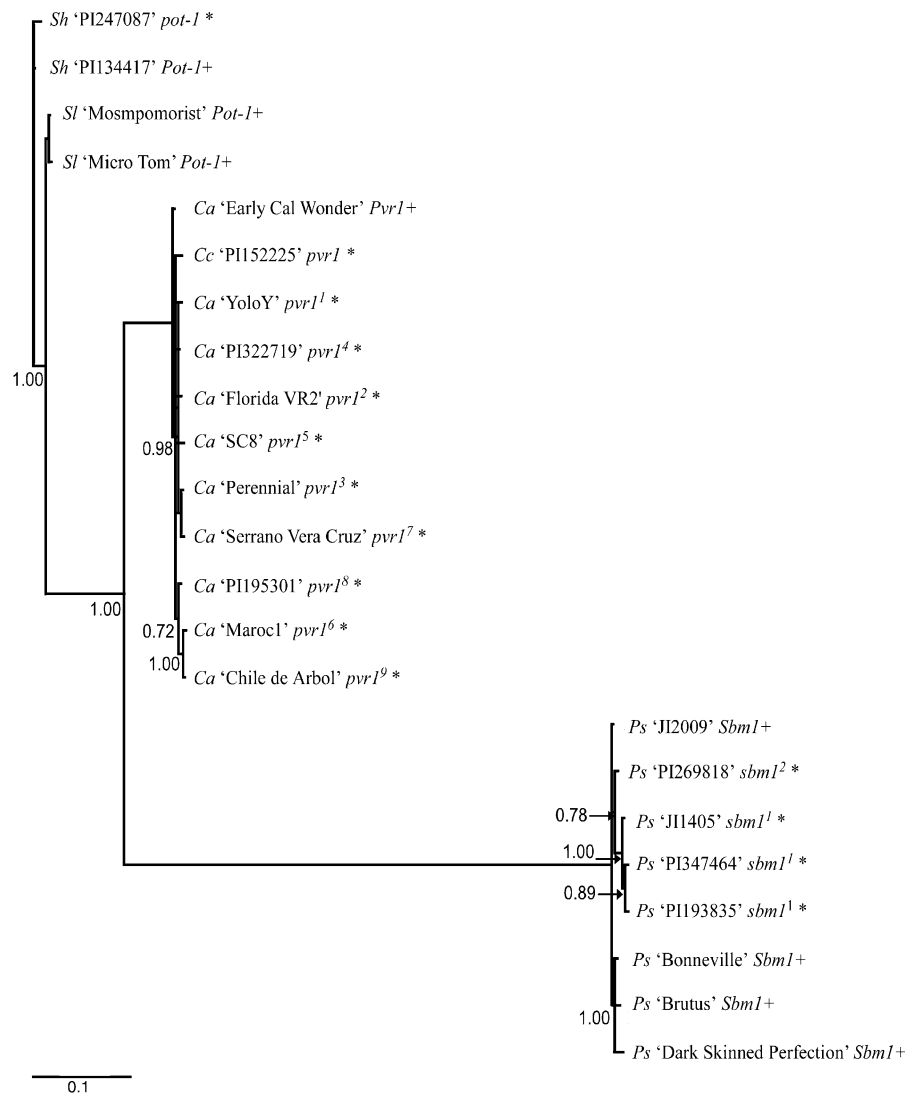
(Charron et al. 2008; Kang et al. 2005a; Ruffel et al. 2002), pea (Gao et al. 2004), and tomato (Ruffel et al. 2005). A total of 23 sequences were used: 11 from pepper, 8 from pea, and 4 from tomato (Fig. 1). Accession numbers are available in Supplementary Table 1. The terminal stop codon was removed, and sequences were aligned by eye using Sequencer v4.8. Three single amino acid insertions existed in *Solanum* relative to *Capsicum* and *Pisum* (Fig. 2). A phylogenetic tree was constructed for use in PAML and HyPhy with the program MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). MrBayes was run with 4 chains for 10<sup>6</sup> generations with a sample frequency of 100 and a 25% burn-in. The analysis was repeated 4 times to make sure that the trees generated were not clustered around local optima. A consensus of all 30,000 trees was generated using PAUP v4.0beta (Swofford 2002). The consensus tree was viewed with TreeView v1.6.6 (Page 1996). The tree was inspected

before continuing with our analysis to ensure that *eIF4E* from the same species grouped together.

### Statistical Analysis Implementation

Average  $\omega$  was measured in DnaSP v4.50 (Rozas and Rozas 1995) using the modified Nei-Gojobori model with Jukes-Cantor correction. Positively ( $dN/dS > 1$ ) and negatively ( $dN/dS < 1$ ) selected sites were detected with the program package PAML version 3.13d (Yang 1997; Yang et al. 2000), using both the M2 and M8 models, and the program package HyPhy (Pond et al. 2005), using the FEL model. PAML compares the maximum likelihood estimators of dN and dS across an alignment to a predefined distribution and uses empirical Bayes methods to identify individual positively selected sites (Nielsen and Yang 1998; Yang et al. 2000). Empirical Bayes may be performed using either the Naïve Empirical Bayes (NEB)

**Fig. 1** MrBayes tree of *eIF4E*. All credibility values >70 are shown. The analysis was run with 4 chains and a burn-in of 25%. The naming system of each allele consists of a two-letter abbreviation of the species, with the genotype indicated in quotes and the allele indicated in italics. Those starting with a capital letter and ending with a '+' are the susceptible wild-type alleles. An asterisk denotes alleles with known resistance against at least one virus strain. Ca = *Capsicum annuum*; Cc = *C. chinense*; Ps = *Pisum sativum*; Sh = *Solanum habrochaites*; Sl = *S. lycopersicum*. The scale bar is in nucleotide substitutions per site (693 nucleotides/sequence)



**Table 1** LRTs comparing models of neutral evolution (M1 and M7) with positive selection (M2 and M8) across *eIF4E*

Model of selection	$l$	$2\Delta l^a$ (df, $p$ )	Estimated parameters <sup>b</sup>
M1: nearly neutral	-2078.8		$p_0 = 0.711$ ; $p_1 = 0.289$ $\omega_0 = 0.067$ ; $\omega_1 = 1$ (fixed)
M2: positive selection	-2075.8	6.0 (2, < 0.0001)	$p_0 = 0.721$ ; $p_1 = 0.246$ ; $p_2 = 0.033$ $\omega_0 = 0.072$ ; $\omega_1 = 1.00$ ; $\omega_2 = 6.85$
Model 7: beta (10 site classes)	-2079.0		Each of 10 $p_0 = 0.1$ $\omega_0 = 0, 0.00052, 0.0054, 0.025, 0.077, 0.18, 0.36, 0.60, 0.83, 0.98$
Model 8: beta & $\omega > 1$ (11 site classes)	-2074.4	9.2 (2, < 0.0001)	Each of 10 $p_0 = 0.095$ ; $p_1 = 0.05$ $\omega_0 = 0.00007, 0.0022, 0.012, 0.034, 0.076, 0.14, 0.25, 0.39, 0.59, 0.84$ ; $\omega_1 = 5.51$

<sup>a</sup> Log-likelihood scores ( $l$ ) were compared for each pair of models (M1 versus M2 and M7 versus M8) using the test statistic  $2\Delta l$ , with significance evaluated from  $\chi^2$  distribution

<sup>b</sup> *eIF4E* coding sequences from *Pisum sativum*, *Solanum lycopersicum*, *S. habrochaites*, *Capsicum annuum*, and *C. chinense* were compared. The proportion of amino acid sites ( $p_n$ ) falling into each selection class ( $\omega_n$ ) was estimated from the data

approach or the Bayes Empirical Bayes (BEB) approach, which accounts for sampling errors in the estimates of model parameters (Yang et al. 2005). Log-likelihood scores were generated in PAML for models of neutral (M1 and M7) and positive (M2 and M8) selection and compared using likelihood ratio tests (LRTs). NEB and BEB posterior probabilities were calculated for sites with  $\omega > 1$  under M2 or M8. HyPhy FEL was run on a computer cluster through the Web-based interface, which is available at <http://www.datamonkey.org> (Pond and Frost 2005).

## Results

The phylogenetic tree generated with MrBayes has high support at major branches and separates *eIF4E* perfectly by species (Fig. 1). Short branch lengths demonstrate a low degree of polymorphism within species. This is interesting given the dramatic differences in phenotypic effects of these alleles, i.e., virus resistance or susceptibility. The total tree length (the sum of all branch lengths) is 0.604. This Bayesian phylogeny was used in the PAML and HyPhy programs to investigate dN/dS ratios that would identify a molecular signature of positive selection.

### Strong Evidence of Positive Selection on the Recessive Resistance Gene *eIF4E*

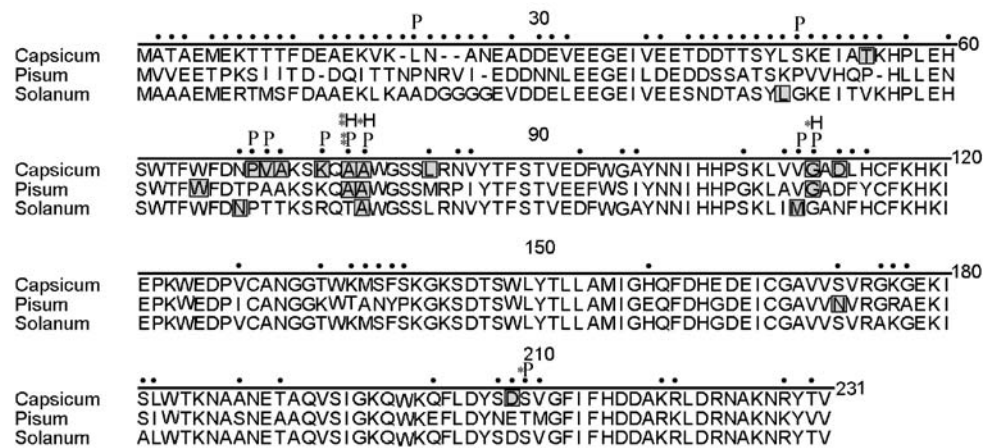
Positive selection is detected statistically as a nonsynonymous-to-synonymous nucleotide-substitution rate ratio ( $\omega$ ) significantly greater than 1. Across our 231 amino acid alignment of 23 complete pepper, tomato, and pea *eIF4E* coding sequences,  $\omega$  is 0.236. This low value is consistent with previous analyses of *eIF4E* across eukaryotes (Athey-Pollard et al. 2002; Gao et al. 2004; Marcotrigiano et al. 1997). An excess of synonymous substitutions is not surprising because many coding sites experience purifying

selection to maintain *eIF4E*'s pleiotropic function of translation initiation. Thus, log-LRTs were used to search for a molecular signature of positive selection acting on sites within the *eIF4E* coding region. Because no a priori expectation exists for the distribution of  $\omega$  values for any given alignment, we compared likelihood values for two pairs of models with different assumed  $\omega$  distributions: (1) M1 (a model of neutral evolution where all sites are characterized by having  $\omega < 1$  or  $\omega = 1$ ) versus M2 (a model of positive selection allowing sites to have  $\omega > 1$ ), which assumes that  $\omega$  values are drawn from a normal distribution and (2) M7 versus M8, models that mirror the evolutionary constraints of M1 and M2 but assume that  $\omega$  values are drawn from a beta distribution (Nielsen and Yang 1998). For each LRT, the model allowing sites to be under positive selection (M2 or M8) fit the *eIF4E* data from potato, pepper, and pea significantly better than the neutral model (M1 or M7) ( $p < 0.0001$ ; Table 1). Under M2, most sites within the *eIF4E* coding sequence experience purifying selection ( $\omega < 72.1\%$ ) or neutral evolution ( $\omega = 1$ ; 24.6%), whereas few codons have a signature of positive selection ( $\omega > 1$ ; 3.3%). However, the mean  $\omega$  value for sites under positive selection is 6.85, nearly a 7-fold excess of nonsynonymous-to-synonymous substitutions, indicating that *eIF4E* has a molecular signature of strong positive selection targeted to a small subset of sites. The proportion of sites falling into each  $\omega$  class was similar under M8 (Table 1).

### Positively Selected Sites Colocalize With Resistance-Determining Amino Acids

Sixteen amino acid sites across the *eIF4E* coding sequence are known to be associated with virus resistance in pepper, tomato, or pea (Fig. 2). The M8 model using both NEB and BEB assigned 10 amino acid sites to the positive selection class, 7 of which were associated with resistance alleles (Fig. 2; Table 2 [heading "A"]). Sites placed into the

**Fig. 2** Alignment of pepper, pea, and tomato. P = sites detected by PAML Model 8 BEB with dN/dS > 1. H = sites detected as being positively selected using HyPhy’s FEL method. A solid circle shows which amino acids are polymorphic between the 3 species. Darkened squares show sites that are polymorphic between virus susceptible and resistant alleles within the associated genera. \* Posterior  $p > 0.9$ . \*\* Posterior  $p > 0.95$



**Table 2** Results of site-specific tests of positive selection

<sup>a</sup> Indicates which codons were identified as being positively selected, the posterior probability, and which species, if any, contain a resistance allele with a polymorphism at that site relative to the wild type  
<sup>b</sup> Indicates a 2 × 2 contingency table showing that the sites identified as positively selected (regardless of posterior probability) by PAML are nonrandomly distributed with respect to resistance-associated amino acids  
 A dash indicates that the amino acid in question is not associated with a resistance allele from any of the plant species considered  
 \* Posterior  $p > 0.9$ ;  
 \*\* Posterior  $p > 0.95$

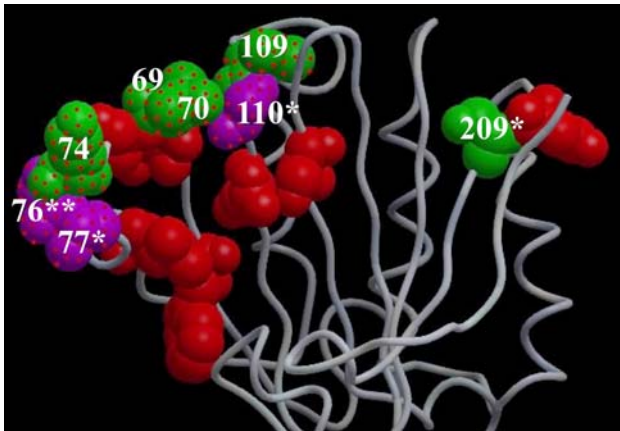
AA position	Model 8 NEB probability	Model 8 BEB probability	FEL probability	Associated with resistance allele in
A <sup>a</sup>				
21	0.84	0.84		–
49	0.70	0.68		–
69	0.64	0.66		<i>Capsicum</i>
70	0.88	0.88		<i>Capsicum</i>
74	0.65	0.65		<i>Capsicum</i>
76	0.99**	0.99**	0.95**	<i>Capsicum, Pisum</i>
77	0.51	0.52	0.91*	<i>Capsicum, Pisum, Solanum</i>
109	0.59	0.59		<i>Solanum</i>
110	0.50	0.52	0.91*	<i>Capsicum, Pisum</i>
209	0.95**	0.94*		–
B <sup>b</sup>				
Results from model 8 BEB	Associated with virus resistance	Not associated with virus resistance		
Positively Selected	7	3		
Not positively selected	9	66		
Fisher exact probability test	$p > 0.001$			

positively selected class are nonrandomly distributed with respect to those involved in virus resistance (Table 2 [heading “B”]; Fisher’s exact test  $p < 0.001$ ).

ML models including positive selection (M2 and M8) fit the data significantly better than their corresponding neutral models (M1 and M7) (Table 1). Therefore, site-specific tests for positive selection using M8 NEB and BEB with PAML and FEL with HyPhy were used to calculate posterior probabilities for each positively selected codon.

All three of the sites identified using FEL with posterior probabilities > 0.9 (sites 76, 77, and 110) colocalize with resistance-determining amino acids (Table 2). Site 76 was identified by NEB, BEB, and FEL and corresponds to an alanine-to-aspartic acid mutation in pepper resistance alleles

*pvr1*<sup>5</sup>, *pvr1*<sup>6</sup>, and *pvr1*<sup>9</sup> (Charron et al. 2008), an alanine-to-aspartic acid mutation in the pea allele *sbm1*<sup>1</sup>, and an alanine-to-proline mutation in the pea allele *sbm1*<sup>2</sup> relative to the wild type (Gao et al. 2004). Site 77 is an alanine-to-aspartic acid mutation in the pepper resistance allele *pvr1*<sup>6</sup> (Charron et al. 2008), an alanine-to-aspartic acid mutation in the pea resistance alleles *sbm1*<sup>1</sup> and *sbm1*<sup>2</sup> (Gao et al. 2004), and an alanine-to-aspartic acid mutation in the tomato resistance allele *pot-1* (Ruffel et al. 2005). Finally, site 110 corresponds to a glycine-to-arginine mutation in pepper resistance alleles *pvr1* from *Capsicum chinense* and *pvr1*<sup>9</sup> from *Capsicum annuum* (Kang et al. 2005a; Charron et al. 2008) and a glycine-to-arginine mutation in the pea resistance allele *sbm1*<sup>1</sup> (Gao et al. 2004).



**Fig. 3** Positively selected sites mapped onto the hypothesized three-dimensional structure reported in Yeam et al. (2007). *Spacefill red* = resistance-associated amino acids. *Spacefill green* = positively selected site identified by PAML. *Spacefill purple* = positively selected site identified by both HyPhy and PAML. *Red stippling* shows sites that are positively selected and resistance associated. The first 60 amino acids are not included because the crystal structure of that region is unresolved. Amino acid positions of positively selected sites are shown in white text. \* Posterior  $p$  (either PAML or HYPHY)  $> 0.9$ . \*\* Posterior  $p$  (either PAML or HYPHY)  $> 0.95$

One of the two codon sites with M8 NEB or BEB posterior probabilities  $> 0.9$  (equivalent to  $p < 0.1$ ; sites 76 and 209) is a resistance-determining amino acid (Table 2). Position 76, discussed previously, is present in several resistance alleles. Although site 209 is not associated with a resistance allele, site 208 directly adjacent is an aspartic acid-to-glycine mutation found in pepper resistance alleles *pvr1<sup>3</sup>*, *pvr1<sup>6</sup>*, *pvr1<sup>7</sup>*, and *pvr1<sup>9</sup>* (Charron et al. 2008).

Using the estimated three-dimensional crystal structure of *Capsicum* eIF4E generated by Yeam et al. (2007), we were able to map positively selected sites on the protein tertiary structure. The sites detected as positively selected cluster in the region of the protein that contains resistance-determining sites and is known interact with the virus (Fig. 3). This region is also involved in binding the 5' cap of the messenger RNA, raising interesting questions regarding maintaining eIF4E's ability to function in host RNA translation initiation (Yeam et al. 2007).

#### HyPhy FEL More Accurately Detects Resistance Sites Than PAML Model 8

Both PAML Model 8 and HyPhy FEL identify positively selected amino acids known to be associated with virus resistance. PAML places 10 amino acids into the positively selected class with  $dN/dS > 1$ , 7 of which are sites involved in virus resistance. Two of the remaining 3 amino acids not known to be involved in virus resistance are directly adjacent to ones that are involved (Fig. 2).

When posterior probabilities are applied using empirical Bayes, only one of the two statistically significant sites detected is a resistance-determining site. HyPhy FEL, in contrast, identifies three sites with posterior probability  $> 0.9$ , all of which are resistance-determining sites (Table 2 and Fig. 2). Considering only the sites with posterior probability  $> 0.9$ , we therefore conclude that although both methods accurately identify functionally significant sites as being under positive selection, HyPhy FEL does so with higher power (three sites identified rather than two) and accuracy (three of three opposed to one of two known resistance sites) for our data set.

## Discussion

### Strong Evidence That the Recessive Resistance Gene eIF4E Is Under Positive Selection

In this study, we showed that the plant resistance gene *eIF4E* is under positive selection based on statistical analyses that consider the rate of accumulation of synonymous and nonsynonymous nucleotide substitutions across an alignment consisting of susceptible and resistant alleles from pepper, tomato, and pea. Positive selection on a recessive resistance gene has never before been documented. Previous studies examining plant defense have focused on genes involved in pathogen detection, intercellular signaling, or genes antagonistic to the pathogen or pest (Tiffin and Moeller 2006). Studies on the evolution of plant defenses have focused largely on gene-for-gene resistance (Stahl and Bishop 2000) but evolution of other positively selected defense genes, including chitinases (Bishop et al. 2000; Tiffin 2004),  $\beta$ -1,2-endoglucanases (Bishop et al. 2005), polygalacturanase inhibitor proteins (Bishop 2005; Stotz et al. 2000), proteinases, proteinase inhibitors, and lectins (Roth and Liberles 2006), has also been investigated. These resistance mechanisms function primarily against bacteria and fungi, are inherited dominantly, and involve the modification of a gene product to fulfill a new role. In contrast, many viral resistance genes are recessively inherited (Kang et al. 2005b; Provvidenti and Hampton 1992; Robaglia and Caranta 2006) and are presumably involved in modifying a host protein to avoid interaction with viral pathogenicity factors (Diaz-Pendon et al. 2004; Fraser 1990). These genes, like *eIF4E*, are typically host factors involved in basic cellular processes that are used by the virus to complete its life cycle. They function as resistance genes pleiotropically once natural mutations arise that prevent viruses from using them. Because they are recessively inherited, natural selection can act on resistance alleles only when they are present in the homozygous state. In this regard it is remarkable that a

signature of positive selection was detected at *eIF4E* given that heterozygotes would not possess a selective advantage even in the presence of viral pathogens.

#### Tests of Positive Selection Accurately Identify Resistance-Determining Codons

The amino acids detected as being positively selected represent one of a few amino acid changes found in resistance alleles relative to susceptible wild-type sequences. Site 76 is one of three amino acid changes in *pvr1*<sup>9</sup> responsible for providing resistance to two strains of *Potato virus Y* (PVY) (Charron et al. 2008). Sites 76 and 77 are two of five and two of three polymorphisms between wild-type pea *eIF4E* and resistance alleles *sbm1*<sup>1</sup> and *sbm1*<sup>2</sup>, respectively (Gao et al. 2004). Similarly, site 77 is one of four amino acid changes in *pvr1*<sup>6</sup> and one of four amino acid changes in resistance allele *pot-1* (Charron et al. 2008). For site 110, we have direct empirical evidence linking this single amino acid specifically with virus resistance. When the glycine-to-arginine mutation was induced at site 110 in susceptible pepper *eIF4E*, the protein–protein interaction with VPg was interrupted and, when expressed transgenically in tomato plants, conferred resistance to three strains of *Tobacco etch virus* (Yeam et al. 2007).

It is interesting that unique mutations in different species tend to be similar among the positively selected sites. In several cases, the specific amino acid change between wild-type and mutant alleles is the same in different species. For instance, site 76 is an alanine in wild-type alleles from both pepper and pea that mutates to aspartic acid in resistance alleles from both species. Site 77 is an alanine-to-aspartic acid mutation in resistance alleles in all three species considered. Finally, the amino acid change at site 110 is a glycine-to-arginine change in resistance alleles from both pepper and pea. This suggests that only particular amino acid changes are sufficient to confer virus resistance. Mutations at those sites that produce other amino acids would tend to be lost by genetic drift or purifying selection if they did not confer a selective advantage by providing resistance to invading viruses.

#### Coevolutionary History of Virus and Host Drive Positive Selection of eIF4E

Positive selection at *eIF4E* is particularly interesting considering that the viral protein VPg, which interacts directly with eIF4E in susceptible host–pathogen combinations (Schaad et al. 2000), is also positively selected, and the specific viral amino acids under positive selection are found in regions of the protein known to be virulence determinants (Moury et al. 2004). The data we present for

positive selection acting within *eIF4E* as well as VPg therefore provides empirical evidence supporting the hypothesis that a coevolutionary “arms race” occurs between host and pathogen in this system (Charron et al. 2008; Dawkins and Krebs 1979). As expected under an “arms race” scenario, multiple resistance alleles with unique resistance spectra and multiple viral strains with unique infectivity are known to exist (Charron et al. 2008; Kang et al. 2005a; Kyle and Palloix 1997; Ruffel et al. 2002). This has been intensively studied in pepper, where nine distinct resistance alleles were investigated for resistance to four viral strains (Charron et al. 2008). Experimental modification of many of the amino acids involved in this protein–protein interaction proves that these sites are directly involved in virus resistance. Positively selected codons of VPg are directly involved in overcoming resistance *eIF4E* alleles (Ayme et al. 2007; Moury et al. 2004). In this study, we found that amino acids involved in host plant resistance are positively selected. The detailed evolutionary knowledge of eIF4E and VPg makes this coevolutionary pathosystem among the best-studied in all of biology.

#### Potential Effect of Positively Selected Sites on Translation Initiation

It is interesting to consider what role positively selected amino acids from eIF4E play in host translation initiation. The region of eIF4E that binds the 5' cap overlaps with the region involved in determining whether the plant is resistant to an invading virus. Sites required for translation initiation are strongly conserved given that recruiting mRNA to the ribosomal complex is an important function of plant cells that must be maintained (Marcotrigiano et al. 1997). When the G110R mutation was induced in wild-type pepper eIF4E, it lost its ability to bind both VPg and the 5' cap (Yeam et al. 2007). In addition, the *pvr1* allele from *Capsicum chinense* that contains this mutation and two others is also unable to bind the 5' cap (Yeam et al. 2007). However, transgenic tomato plants containing pepper *eIF4E* with this mutation alone exhibit no obvious abnormalities (Yeam et al. 2007). In addition, homozygous *pvr1* pepper plants do not appear to exhibit any negative phenotypic effects. Either the mutated form of eIF4E is still able to participate in translation initiation despite not being able to bind to the 5' cap in vitro, or the presence of *eIF4E* paralogs are responsible for reducing the evolutionary constraints of this locus. Under the latter scenario, paralogs would take over the cap-binding function while the version of *eIF4E* involved in resistance is allowed to diverge (Robaglia and Caranta 2006). However, because nine other known resistance alleles in pepper are able to complement an *eIF4E* knockout mutant in yeast, it seems more likely

that translation initiation function continues in resistance alleles (Charron et al. 2008).

### HyPhy FEL Detects Resistance Sites More Accurately and With Higher Power

Identification of positively selected genes does not provide detailed understanding of adaptive change. We contest that several additional pieces of information are required. First, the area under positive selection must be further localized to specific amino acids. Statistical procedures have been developed for this purpose (Massingham and Goldman 2005; Nielsen and Yang 1998; Suzuki and Gojobori 1999). These techniques provide information on the physical distribution of molecular evolution that may be used to better understand selective forces acting on a gene (Anisimova et al. 2002). In addition, changes in particular amino acids must be correlated with an associated change in phenotype (Golding and Dean 1998). In this study, we attempted to fulfill these criteria to confidently connect positive selection with causal forces. We have used popular statistical methods to identify site-specific positive selection of a well-studied resistance gene in plants. Both methods identified a small number of positively selected amino acid sites associated with resistance alleles. Our findings indicate that both PAML Model 8 and HyPhy FEL accurately and precisely pinpoint single amino acids that are known to function in resistance alleles to interrupt the virus life cycle. However, HyPhy FEL does so with greater power and precision because all three amino acids predicted to be positively selected are associated with virus resistance. We conclude, therefore, that HyPhy FEL is an appropriate method for similar data sets to identify sites having biologic relevance to the fitness of the host.

### Conclusion

Interaction with translation initiation factors appears to be a common infection strategy for viruses that infect plants (Schaad et al. 2000). Amino acid changes in eIF4E that result in resistance to a number of viral families have evolved independently in a variety of plant taxa (Charron et al. 2008; Gao et al. 2004; Kang et al. 2005a; Morales et al. 2005; Nicaise et al. 2003; Nieto et al. 2006; Ruffel et al. 2002, 2005; Wicker et al. 2005). The results of this study provide some insight into the driving evolutionary forces responsible for this process. In addition, our results support the use of ML and Bayesian methods for detecting site-specific positive selection, particularly in natural populations where the fitness consequences of single amino acid changes cannot be demonstrated experimentally. In conclusion, we suggest that the accurate identification of amino

acids with dramatic biologic consequences in this system make these methods a powerful predictive tool for targets of genetic crop improvement for a number of agronomically important traits.

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