Molecular Evolution of the Wound-Induced Serine Protease Inhibitor *wip1* in *Zea* and Related Genera

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Plant defense mechanisms have been the subject of intensive investigation. However, little is known about their long-term evolutionary dynamics. We investigated the molecular diversity of a wound-induced serine protease inhibitor, *wip1,* in the genus *Zea,* as well as the divergence of *wip1* among four genera, *Zea, Tripsacum, Sorghum,* and *Oryza,* in order to gain insight into the long-term evolution of plant defense. The specific objectives of this study were to determine (1) whether *wip1* has a history of positive or balancing selection, as has been shown for genes involved in plant defense against pathogens, and (2) if the evolutionary histories of *wip1* inhibitory loops, which come into closest contact with proteases, differ from the evolutionary history of other parts of this gene. The *Zea* polymorphism data are consistent with a neutral evolutionary history. In contrast, relative-rate tests suggest a nonneutral evolutionary history. This inconsistency may indicate that selection acting on *wip1* is episodic or that *wip1* evolves in response to selection favoring novel alleles. We also detected significant heterogeneity in the evolutionary rates of the two inhibitory loops of *wip1*—one inhibitory loop is highly conserved, whereas the second has diverged rapidly. Because these two inhibitory loops are predicted to have very similar biochemical functions, the significantly different evolutionary histories suggest that these loops have different ecological functions.

Introduction

Plants have a complex array of proteins, morphological traits, and secondary chemicals that defend them against attack by herbivores and pathogens. Short-term field studies have shown that herbivores and pathogens may impose selection on these traits (Simms and Rausher 1989; Mauricio and Rausher 1997; Schonle and Bergelson 2000). However, patterns of selection detected during short-term studies do not necessarily reflect long-term evolutionary dynamics, and relatively little is known about the long-term evolutionary dynamics of defense genes. Molecular population genetic analyses of defense genes offer an opportunity to examine these evolutionary dynamics and to determine the role selection has played in the evolution of defense genes.

There are two basic theoretical models that predict how defense genes evolve (Stahl et al. 1999). One of these models predicts that variation in defense mechanisms is transitory because of positive selection imposed by parasites (Dawkins and Krebs 1979). If this model accurately describes the evolution of plant defense genes, then these genes should contain low levels of genetic diversity, and population genetic analyses should reveal evidence of repeated positive selection. An alternative model predicts that variation in defense genes is maintained for long periods as a consequence of frequency-dependent selection associated with fluctuations in the frequencies of parasite genotypes (Jayakar 1970; Clarke 1976; May and Anderson 1983; Seger 1988; Frank 1992) or costs associated with the expression of defense (Roy and Kirchner 2000; Tiffin 2000). Under these models, defense genes should contain above-average levels of diversity, and this diversity should be spread between two, or possibly more, allelic classes whose most recent common ancestor is older than expected under a null model of neutral evolution.

Several molecular studies provide evidence that genes involved in pathogen recognition (i.e., R-genes) are subject to selection (Parniske et al. 1997; Meyers et al. 1998; Wang et al. 1998). However, few studies have examined intraspecific diversity at individual resistance loci. Two investigations of intraspecific polymorphism of R-genes in *Arabidopsis thaliana* revealed patterns of polymorphisms that were consistent with selection having acted on these genes. One of these investigations detected strong evidence that the *rpm1* gene had evolved in response to balancing selection, presumably resulting from fluctuations in selective pressure imposed by parasites (Stahl et al. 1999). Results from the other study, which examined intraspecific polymorphism at the *rps1* locus, were less clear but also suggestive of a nonneutral evolutionary past, perhaps also the result of some form of balancing selection (Caicedo, Schaal, and Kunkel 1999).

Although these studies have begun to provide insight into the evolution of genes involved in defense against pathogens, they have focused primarily on just one aspect of defense—plant recognition of pathogens. Pathogen recognition can cause hypersensitive response and increased expression of a host of genes (i.e., induced defenses); however, R-genes themselves do not actually prevent or retard pathogen infection (Somssich and Hahlbrock 1998). Few studies have examined the molecular evolution of the genes that code for proteins that actually limit the severity of parasite attack (but see Bishop, Dean, and Mitchell-Olds 2000).

Protease inhibitors (PIs) are among the best-studied plant defenses not encoded by R-genes (Garcia-Olmedo et al. 1987; Ryan 1990). These inhibitors are thought to be involved primarily in defense against herbivores, which rely on proteases to digest the proteins they con-

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sume (Ryan 1990; Koiwa, Bressan, and Hasegawa 1997). However, because pathogens rely on proteases to facilitate infection and spread within hosts, PIs may also limit the severity of pathogen infection (Ryan 1990). Protease inhibitors may also be involved in the regulation of the plants' own proteases, especially in seeds where PIs can be found in high concentrations and may prevent the untimely degradation of seed storage proteins (Koiwa, Bressan, and Hasegawa 1997). Several lines of empirical evidence support a role for protease inhibitors in plant defense: the expression of many PI genes is induced following mechanical or herbivore damage (Green and Ryan 1972; Koiwa, Bressan, and Hasegawa 1997), herbivores grow more slowly when reared on artificial diets containing PIs than when reared on artificial diets without PIs (Jongsma and Bolter 1997), and transgenic plants expressing elevated levels of PIs incur less herbivore damage than control plants (Hilder et al. 1987; Johnson et al. 1989; McManus, White, and McGregor 1994).

Protease inhibitors function as specific substrates for the proteases they inhibit. However, unlike normal substrates, which are cleaved by proteases and released quickly, the PI-protease complex is stable, and proteolysis of the inhibitor is limited and extremely slow (Laskowski 1985; Garcia-Olmedo et al. 1987). The specificity and efficacy of inhibition are determined by the degree of stereochemical complementation between the protease active site and a short inhibitory loop that extends out from the main body of the inhibitor molecule (Bode and Huber 1992).

Molecular analyses of animal PIs have revealed some evidence for rapid evolution of these inhibitory loops. In particular, the reactive centers of homologous PIs isolated from related species are hypervariable (Hill et al. 1984; Laskowski et al. 1987; Creighton and Darby 1989), and the reactive centers of duplicated PI genes diverge rapidly (Hill and Hastie 1987). Although these observations have been interpreted as evidence for positive selection, presumably in response to selective pressure imposed by parasites (Hill and Hastie 1987; Laskowski et al. 1987; Creighton and Darby 1989), little is known about the allelic diversity at specific PI loci.

In order to further our understanding of the molecular evolution and population genetics of plant protease inhibitors and plant defense mechanisms in general, we investigated the molecular diversity of the *wip1* gene, a wound-induced serine protease inhibitor, in four taxa within the genus *Zea.* We also analyzed divergence of *wip1* homologs isolated from species within the genera *Zea, Tripsacum, Sorghum,* and *Oryza. Wip1* codes for a wound-induced protein with high similarity to members of the Bowman-Birk family of serine PIs (Eckelkamp, Ehmann, and Schopfer 1993; Rohrmeier and Lehle 1993). Like most Bowman-Birk PIs, *wip1* is predicted to have two inhibitory domains (Rohrmeier and Lehle 1993). However, unlike most Bowman-Birk PIs that inhibit both trypsin and chymotrypsin proteases (Ikenaka and Norioka 1986), both inhibitory regions in *wip1* are predicted to inhibit chymotrypsin proteases (Rohrmeier and Lehle 1993). The specific objectives of this study were to determine (1) whether *wip1* has a history of positive or balancing selection and (2) whether the evolutionary history of *wip1* inhibitory loops differs from the evolutionary history of other parts of this gene.

Table 1
Species Names, USDA Accessions, and Sample Numbers Used in this Study and a List of GenBank Accession Numbers

Accession	Sample No.	GenBank Nos.
Zea mays ssp. *mays*		
Chillo	*mays* 1a	AF396265
Chulpi chico	*mays* 2a, 2b	AF396267, AF396266
Confite puneneo	*mays* 3a	AF396268
Enano	*mays* 4a, 4b	AF396269, AF396270
Montana	*mays* 5a	AF396271
Morano	*mays* 6a, 6b	AF396272, AF396273
Pira	*mays* 7a, 7b	AF396274, AF396276
Cuban Flint (Cub 65 Cimyt)	*mays* 8a	AF396275
Zea mays ssp. *mexicana*		
PI 384060	*mex* 1a	AF396286
PI 566673	*mex* 2a	AF396287
PI 566680	*mex* 3a	AF396288
PI 566681	*mex* 4a	AF396289
PI 566683	*mex* 6a	AF396290
PI 566685	*mex* 7a, 7b	AF396292, AF396291
PI 566688	*mex* 8a	AF396293
PI 566691	*mex* 9a, 9b	AF396295, AF396294
Zea mays ssp. *parviglumis*		
PI 331783	*parv* 1a	AF396301
PI 331785	*parv* 3a	AF396302
PI 331786	*parv* 4a	AF396298
PI 331788	*parv* 5a, 5b	AF396303, AF396304
PI 351707	*parv* 6a, 6b	AF396305, AF396306
PI 384061	*parv* 7a, 7b	AF396307, AF396308
PI 384062	*parv* 8a, 8b	AF396309, AF396310
M 046	*parv* 10a	AF396296
M 063	*parv* 11a, 11b	AF396297, AF396300
M 106	*parv* 12a	AF396299
Zea luxurians		
M 111	*lux* 1a	AF396285
PI 21863	*lux* 2a	AF396277
PI 21877	*lux* 3a	AF396278
PI 30919	*lux* 4a, 4b	AF396279, AF396280
PI 306615	*lux* 5a, 5b	AF396282, AF396281
PI 306617	*lux* 6a, 6b	AF396283, AF396284

Materials and Methods

Sampling DNA Sequences

We PCR-amplified approximately 660 bp of *wip1* from eight accessions of *Zea mays* ssp. *mays,* eight accessions of *Zea mays* ssp. *mexicana,* 10 accessions of *Zea mays* ssp. *parviglumis,* six accessions of *Zea luxurians,* and one accession of *Tripsacum dactyloides* (table 1). PCR conditions for all templates were 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C (forward: 5′-tgcgagctagacaacatcatgcgcc-3′; reverse: 5′-agggcgcggccacgttctcc-3′). The PCR primers amplified both exons (327 bp in *Zea*) and one intron (90–95 bp in *Zea*) of *wip1* and ~240 bp of flanking DNA (fig. 1). Sequences for *Oryza sativa* and *Sorghum bicolor wip1* homologs were obtained from GenBank (accession numbers AP002526 [*O. sativa*] and AW680689 [*S. bi-*

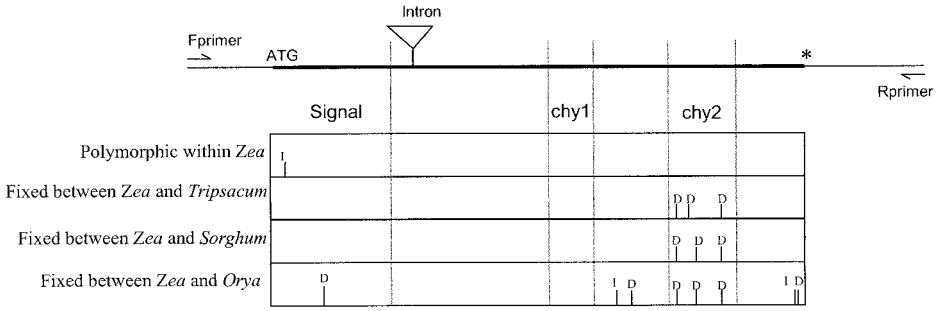

FIG. 1.—*Wip1* gene structure and distribution of insertions and deletions that are polymorphic within *Zea* and fixed between *Zea* and *Tripsacum dactyloides, Zea* and *Sorghum bicolor, Zea* and *Oryza sativa1,* and *Zea* and *O. sativa2.* In *Zea,* approximately 660 bp were amplified (the coding region is 327 bp; the intron ranged from 90 to 95 bp).

color]). A BLAST search (Altschul et al. 1990) of GenBank data revealed two *O. sativa* sequences within 2,500 bp of one another, and both showed significant similarity to *wip1* ($P < 1 \times e^{-41}$). For this study, these sequences were labeled *O. sativa1* (bp 100073–99158) and *O. sativa2* (bp 102325–101834).

More than half of the alleles identified after the initial collection of data contained one or more unique single-base-pair variants (or ''singletons'') relative to the remainder of the sequences. Singletons from individual cloned products can represent either true sequence variation or polymerase error. Therefore, we reamplified and resequenced all alleles containing singletons in order to determine which singletons were true variants. Approximately one third of the singletons in the initial data set were confirmed by this strategy. We assumed that the other 21 singletons resulted from polymerase error and excluded these from the analyses. Our estimated rate of polymerase error of ≈1 in 1,350 bp is similar to previously reported polymerase error for *Zea* DNAs (Eyre-Walker et al. 1998; Tiffin and Gaut 2001). Singleton checking, which required the reamplifying and resequencing of a majority of the alleles, detected no evidence for PCR recombinants. All sequences have been submitted to GenBank (table 1).

Sequence Analyses

Genealogies were constructed with PAUP* 4.0b (Swofford 1998) using the neighbor-joining method (Saitou and Nei 1987) with the HKY85 (Hasegawa, Kishino, and Yano 1985) genetic distance. A sequence from *T. dactyloides* was used as an outgroup. Data were resampled 1,000 times for bootstrap analyses. All nucleotide sites were used for genealogical reconstruction. Estimates of genetic diversity, θ (Tajima 1983) and π (Watterson 1975), were calculated separately on silent (synonymous and intron sites), synonymous, and nonsynonymous sites. Evidence for nonneutral evolution was investigated using the tests of Fu and Li (1993), Tajima (1989), Sawyer, Dyjhuizen, and Hartl (1987), and McDonald and Kreitman (1991) (MK). All measures of polymorphism and tests of neutral evolution were calculated using DnaSP, version 3.5 (Rozas and Rozas 1999).

Relative-rate tests between sequences from different genera were conducted using the method of Fitch (1976) and Tajima (1993) as implemented by MEGA (Kumar et al. 2000). Rate tests on *Zea* were conducted with the *Z. mays* ssp. *mays* 4b allele. This allele was chosen because it is located in the approximate middle of the *wip1* genealogy and preliminary analyses showed that results obtained with this allele were typical of results obtained with other *Zea* alleles. Results from the rate tests were similar when either the *O. sativa1* or the *O. sativa2* sequence was used as the outgroup, and only results obtained with the *O. sativa2* sequence are presented. All analyses involving *O. sativa* sequences were conducted on two alignments that differed in the location of an indel. Results from the tests differed little, and only results from the alignment that minimized the number of indels are presented.

Distribution of Changes in Different Gene Regions

A series of contingency tests were used to determine if polymorphic sites within *Zea* and fixed differences between *Zea, T. dactyloides, S. bicolor,* and *O. sativa* were distributed heterogeneously among four regions into which the *wip1* sequence was divided a priori. Three of these four regions were predicted to have functional significance: two reactive-site loops active against chymotrypsin (designated chy1 and chy2) and a putative secretion signal sequence that is cleaved to form the mature protein (Rohrmeier and Lehle 1993). The fourth region included all parts of the coding region that were not part of the above regions and is hereafter referred to as the structural region. The boundaries of the reactive-site loops were defined by the cysteine residues that are conserved across the Bowman-Birk family of inhibitors; these cysteines were included as part of the structural region of the molecule. The significance levels of the 4-by-2 contingency tests were evaluated using a χ^2 statistic, and the significance levels of the 2-by-2 tests were evaluated using Fisher's exact tests.

Results

Molecular Diversity and Evidence for Nonneutral Evolution

A sample of 46 *wip1* alleles from four taxa of the genus *Zea* (*Z. mays* ssp. *mays, Z. mays* ssp. *mexicana,*

Table 2
Number of Sequences (*N*), Number of Haplotypes (*H*), Number of Segregating Sites (*S*), and Measures of Genetic Diversity Calculated on Silent (θ_{silent} and π_{silent}), Synonymous (θ_S and π_S), and Nonsynonymous (θ_N and π_N) Sites

Gene	Species	*N*	*H*	*S*	θ_{silent}	π_{silent}	θ_S	π_S	θ_N	π_N
wip1	*Zea mays* spp. *mays*	12	7	9	0.021	0.025	0.014	0.017	0.0090	0.0074
	Zea mays spp. *mexicana*	9	5	9	0.030	0.030	0.026	0.024	0.0067	0.0076
	Zea mays spp *parviglumis*	14	9	11	0.026	0.026	0.013	0.019	0.0097	0.0058
	Zea luxurians	9	6	7	0.017	0.019	0.011	0.010	0.0083	0.0076
adh1	*Zea mays* ssp. *mays*	7	5	9	0.021	0.023	0.023	0.028	0.0010	0.0007
	Zea mays ssp. *parviglumis*	8	7	19	0.028	0.028	0.052	0.054	0.0010	0.0014
	Zea luxurians	7	5	9	0.012	0.012	0.027	0.031	0.0000	0.0000
glb1	*Zea mays* ssp. *mays*	7	7	33	0.026	0.027	0.040	0.044	0.0129	0.0122
	Zea mays ssp. *parviglumis*	8	8	47	0.043	0.037	0.043	0.047	0.0174	0.0142
	Zea luxurians	6	5	11	0.015	0.016	0.013	0.011	0.0051	0.0043
waxy	*Zea mays* ssp. *mays*	15	7	9	0.014	0.013	0.014	0.008	0.0006	0.0006
	Zea mays ssp. *mexicana*	8	7	12	0.015	0.013	0.020	0.016	0.0021	0.0019
	Zea mays ssp. *parviglumis*	9	9	11	0.019	0.016	0.019	0.014	0.0020	0.0018
	Zea luxurians	9	5	4	0.008	0.009	0.006	0.007	0.0007	0.0004

NOTE.—*S* and *H* were calculated on coding regions only. Approximate total lengths of the genes were as follows: *wip1*, 660 bp; *adh1*, 1,421 bp; *glb1*, 1,285 bp; *waxy*, 1,275 bp.

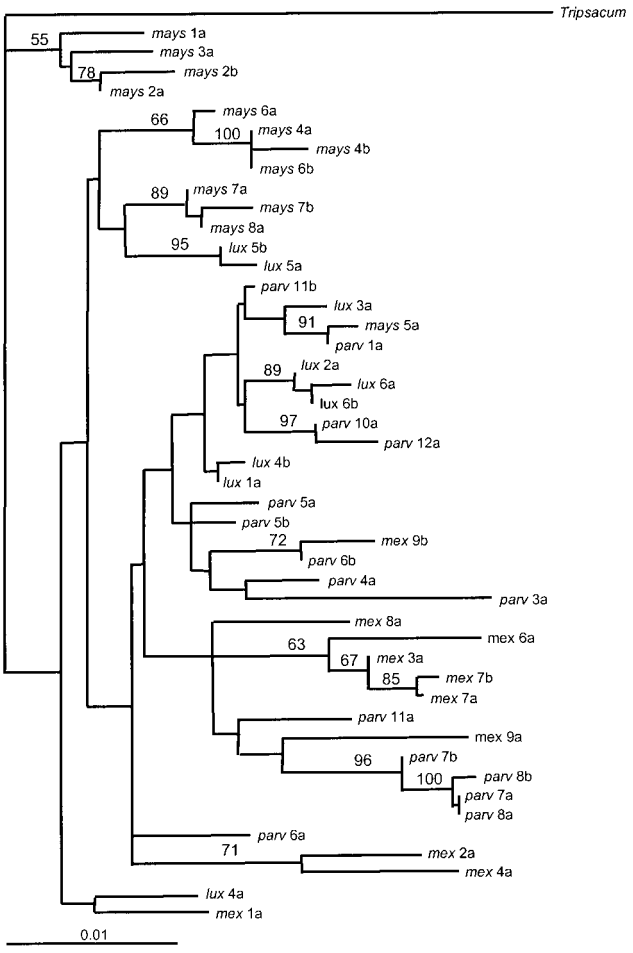

FIG. 2.—Neighbor-joining reconstruction of the genealogical relationships among *Zea wip1* alleles. A *Tripsacum dactyloides* sequence was used as an outgroup. Bootstrap values for nodes supported in >50% of 1,000 bootstrap replicates are shown above the branches. Allele names are presented in table 1.

Z. mays ssp. *parviglumis*, and *Z. luxurians*) revealed similar levels of genetic diversity in *wip1* among the four taxa (table 2). Levels of genetic diversity were also within the range of diversity found in other apparently neutrally evolving *Zea* nuclear genes: *adh1*, *glb1*, and *waxy* (Gaut and Clegg 1993; Hilton and Gaut 1998) (table 2). If *wip1* evolved in response to balancing or positive selection, *wip1* would be expected to have more or less diversity, respectively, than neutrally evolving loci. The similar levels of diversity found in *wip1*, *adh1*, *glb1*, and *waxy* suggest that *wip1* has a neutral evolutionary history within the genus *Zea*.

The genealogical relationships among *wip1* alleles (fig. 2) are also similar to the genealogies of these other neutrally evolving nuclear loci. In particular, branches in the genealogy are generally poorly supported and offer little phylogenetic signal (e.g., Tiffin and Gaut 2001). Consistent with the lack of phylogenetic signal, there were no fixed differences among the *wip1* alleles isolated from the *Z. mays* subspecies or between any of these subspecies and *Z. luxurians*. There were, however, numerous shared polymorphisms among the four taxa.

The genealogy also provides no evidence for well-supported branches that would be indicative of balancing selection having maintained different classes of *wip1* alleles. Similarly, Tajima's (1989) and Fu and Li's (1993) tests (table 3), both of which test the frequency of rare to common intraspecific polymorphisms to infer nonneutral evolutionary history, were not significant.

Table 3
Results from Four Tests of Neutral Evolution: Tajimas *D*, Fu and Li's *D and *F**, and McDonald-Kreitman (MK) tests**

Species	*D*	*D**	*F**	MK[a]
Zea mays ssp. *mays*	−0.28	−0.76	−0.72	0.08
Zea mays ssp. *mexicana*	0.12	0.62	0.56	0.53
Zea mays ssp. *parviglumis* . . .	−0.43	−0.15	−0.26	0.06
Zea luxurians	−0.32	−0.54	−0.54	0.12

[a] *P* values (Fisher's exact test) using a sequence from *Tripsacum dactyloides* as the reference sequence.

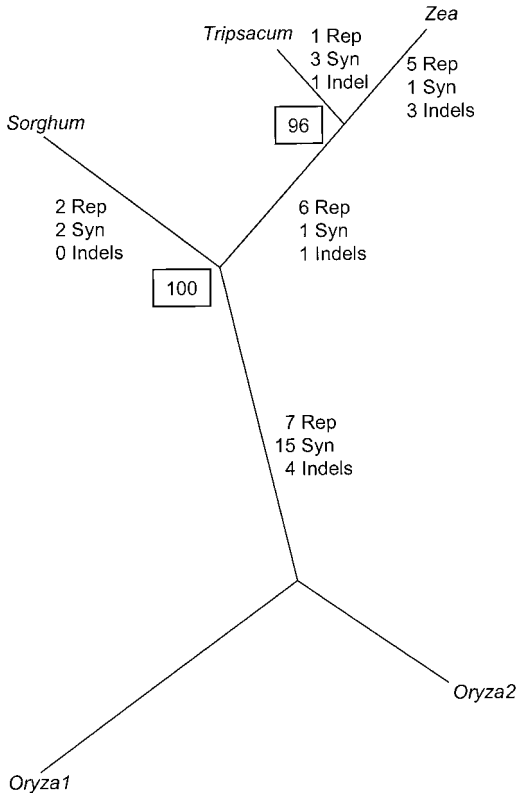

FIG. 3.—Neighbor-joining reconstruction of the genealogical relationships among *wip1*-like sequences from *Zea, Tripsacum dactyloides, Sorghum bicolor,* and *Oryza sativa.* Bootstrap support for the nodes of the tree are presented in boxes next to the nodes. Branch lengths are proportional to the numbers of nucleotide substitutions that differentiate the taxa. The numbers of replacement (rep), synonymous (syn), and insertion-deletion (indels) that differentiate *Zea, T. dactyloides, S. bicolor,* and *O. sativa2* sequences are presented next to the branches. Due to a deletion in *T. dactyloides,* the placement of two replacement changes and one indel that differentiate *Zea* from *S. bicolor* and *O. sativa* is ambiguous, and these changes could have occurred before the divergence of *Zea* and *T. dactyloides.*

Likewise, Sawyer, Dyjhuizen, and Hartl's (1987) tests, which compare the frequency of rare to common synonymous and replacement polymorphisms within a taxon, were not significant (all $P > 0.24$; data not shown), and MK tests, which compare the frequencies of intraspecific polymorphisms to fixed differences between related species were also not significant (table 3). In order to increase our sample size, we also conducted these tests of neutral evolution with the data pooled from all four *Zea* taxa; these tests were also not significant (data not shown). Overall, the results from these tests were largely consistent with the *wip1* gene having evolved neutrally within *Zea.*

Relative-Rate Tests

In addition to the population analyses, we examined the molecular evolution of *wip1*-like genes among four genera in the Poaceae family. In particular, we used relative-rate tests (Tajima 1993) to test for heterogeneity in the rate at which *wip1*-like sequences have diverged between *Zea* and *T. dactyloides, Zea* and *S. bicolor,* and *T. dactyloides* and *S. bicolor.* Significant rate heterogeneity is of interest because it may indicate differences in the selective environment experienced by *wip1*-like genes in these genera (Gillespie 1986; Kreitman and Akashi 1995). Relative-rate tests indicate that *wip1* has evolved significantly faster in the lineage leading to *Zea* than in the lineage leading to *S. bicolor* (fig. 3 and table 4). The test comparing *wip1* in *Zea* with the *wip1*-like sequence from *S. bicolor* was significant ($P < 0.05$), and the test comparing the *wip1*-like sequences from *T. dactyloides* and *S. bicolor* was marginally significant ($P = 0.052$). In contrast, there was no difference in the relative evolutionary rates of *wip1* between *Zea* and *T. dactyloides* (four unique changes in each branch; $P = 1.0$).

Significant evolutionary rate heterogeneity may result from differences in demographic histories and mutation rates as well as selective forces (Gillespie 1984, 1986; Hudson, Kreitman, and Aguade 1987). Because

Table 4
Results from Relative-Rate Tests Comparing the Divergence of Nine Genes from *Zea mays* ssp. *mays* and *Sorghum bicolor* Using *Oryza sativa* as an Outgroup

Gene[a]	Sequence Length (bp)	No. of Changes Unique to *Zea*	No. of Changes Unique to *S. bicolor*	No. of Changes Unique to *O. sativa*	*P* Value of Relative-Rate Test
Wip1	285	12	4	22	0.046
NADPH-dependent reductase	332	7	4	39	0.37
ADP-glucose pyrophosphorylase	221	0	8	12	0.005
MADS box transcription factor	554	9	5	57	0.29
MADS box protein (ZAP1)	519	7	8	43	0.80
Plasma membrane MIP protein	353	4	4	19	1.0
PhytochromeC	311	10	10	36	1.0
Alcohol dehydrogenase1 (*adh1*)	581	17	14	49	0.59
Starch branching synthase (*waxy*)	729	15	15	71	1.0

[a] Gene names are those given for the *Zea* sequence in GenBank. GenBank accession numbers for the sequences used in the tests (accession numbers for each gene are given in the order *Zea, S. bicolor, O. sativa*): NADPH-dependent reductase (U50275, U87454, U87451), ADP-glucose pyrophosphorylase (M81603, AF010283, U66041), MADS box transcription factor (Y09302, U49734, U78892), MADS box protein (ZAP1) (L46400, U32110, AB00325), plasma membrane MIP protein (*pip1-2*) (AF131201, U87981, AF022737), phytochromeC (*phyC*) (U61220, U56731, AF141942), alcohol dehydrogenase1 (*adh1*) (X00580, AF124045, X16296), starch branching synthase (*waxy*) (U23945, X62134), *wip1* (AF396270, AW680689, AP002526).

demographic histories and mutation rates are expected to affect entire genomes, if these forces are responsible for the significant rate heterogeneity detected in *wip1*, we would expect that nuclear genes commonly exhibit significantly faster evolutionary change in *Zea* than in *S. bicolor*. To test this possibility, we used relative-rate tests to compare the evolutionary rates of eight genes for which sequence data from *Z. mays* ssp. *mays*, *S. bicolor*, and *O. sativa* were available from GenBank. Relative-rate tests conducted on these eight genes revealed no evidence for a genomewide evolutionary rate increase in *Zea* compared with *S. bicolor* (table 4). In fact, one of these genes (an ADP-glucose pyrophosphorylase) has evolved significantly faster in the *S. bicolor* lineage than in the *Zea* lineage. These eight rate tests were conducted only after significant rate heterogeneity was detected for *wip1*, and therefore the significance of the *wip1* test was not adjusted for multiple comparisons.

One caveat regarding these tests is that we do not know if these genes are duplicated, and thus we cannot be certain that tests were conducted on orthologs rather than paralogous gene copies. However, the pattern of divergence among sequences from the three taxa was what was expected for orthologous gene copies; i.e., the sequences from *Zea* and *S. bicolor* were more similar to one another than either was to either of the *O. sativa* sequences. Moreover, the genealogies of *wip1*-like genes had 100% bootstrap support for a branch containing *O. sativa1* and *O. sativa2* (see fig. 3), suggesting that the duplication event that resulted in two *wip1*-like sequences in *O. sativa* occurred after the divergence of *Oryza* from *Zea*, *Tripsacum*, and *Sorghum*. Taken together, these data suggest that we are making valid comparisons, and the relative-rate tests provide insight into the relative evolutionary rates of genes in *Zea* and *S. bicolor*.

The significance of the relative-rate tests was largely due to changes at replacement sites, providing further evidence for a role of selection in causing the significant rate heterogeneity in *wip1*. Synonymous-site changes were distributed on the *Zea* and *S. bicolor* branches with approximately equal frequencies (2 vs. 3 changes in the *Zea* and *S. bicolor* branches, respectively), whereas changes at replacement sites were heavily concentrated on the *Zea* branch (10 vs. 2 changes in the *Zea* and *S. bicolor* branches, respectively). Indels were also concentrated on the *Zea* branch (3 vs. 0 insertions in *Zea* and *S. bicolor*, respectively).

Distribution of Fixed and Polymorphic Sites in Different Gene Regions

Four-by-two contingency tests revealed no evidence (all $P > 0.5$) that polymorphic sites within *Zea* taxa are distributed heterogeneously among the four regions into which we a priori divided the *wip1* gene. In contrast, the concentrations of both nucleic acid changes and indels that differentiated *Zea*, *T. dactyloides*, *S. bicolor*, and *O. sativa* differed significantly among the four regions (table 5). Contingency tests conducted on synonymous and replacement sites separately revealed no evidence for significant heterogeneity in the distribution of synonymous sites (table 5), but the distribution of replacement sites was highly heterogeneous (table 5). Moreover, the ratio of replacement to synonymous substitutions was significantly heterogeneous, with the chy2 region having the highest ratio of K_a to K_s (table 5). A series of 2-by-2 contingency tests suggested that the significant heterogeneity detected by the 4-by-2 contingency tests was largely due to a greater frequency of evolutionary changes occurring in the chy2 region relative to the chy1 region and the structural regions (table 5). All of the 2-by-2 contingency tests remained significant after a sequential Bonferroni correction for the six comparisons that were conducted within each class of changes. The 4-by-2 tests comparing the distributions of insertions and deletions and synonymous to replacement changes were not significant after a sequential Bonferroni correction for multiple tests (Rice 1989); however, the probability that four of six tests were significant at $P < 0.05$ was very low.

Relative-rate tests also indicated that the chy2 region in *Zea* has diverged rapidly from the chy2 regions in *S. bicolor* and *T. dactyloides*. If amino-acid-changing indels were included in the divergence data, tests comparing the relative evolutionary rates of the chy2 region in *Zea* to *S. bicolor* and of *T. dactyloides* to *S. bicolor* were both significant (7 changes vs. 1 change in *Zea* and *S. bicolor*, respectively [$P < 0.05$], and 4 changes vs. 0 changes in *T. dactyloides* and *S. bicolor*, respectively [$P < 0.05$]).

Discussion

Evolutionary History of *wip1*

Intraspecific and intergeneric analyses revealed different pictures of the evolutionary history of the wound-induced serine protease inhibitor *wip1*. The genetic diversity of *wip1* from *Zea* taxa and the genealogical relationships among *wip1* alleles are typical of other *Zea* nuclear loci. Moreover, tests of neutrality, including the tests of Tajima (1989), Fu and Li (1993), Sawyer, Dyjhuizen, and Hartl (1987), and McDonald and Kreitman (1991), are not significant. Thus, the results of tests that rely on intraspecific polymorphism data are consistent with a neutral evolutionary history of *wip1*. In contrast, relative-rate tests reveal that *wip1* has evolved significantly faster in the lineage leading to *Zea* than in the lineage leading to *S. bicolor*. The faster rate of evolutionary change was particularly pronounced in one of the two inhibitory loops which come into close contact with proteases during inhibition and were a priori expected to be possible targets of selection.

Although significant rate heterogeneity may result from nonselective forces including changes in mutation rates, life history, or effective population sizes (Gillespie 1986; Kreitman and Akashi 1995), our evidence argues against this explanation for the significant rate heterogeneity in *wip1*. Unlike changes in selective pressure, these neutral evolutionary forces should affect entire genomes (Hudson, Kreitman, and Aguade 1987). How-

Table 5
Four-by-Two Contingency Tests Comparing the Distributions of Polymorphic Sites Within *Zea* and Total Sites, Synonymous Sites, Replacement Sites, and Indels that Differentiate *Zea, Tripsacum dactyloides, Sorghum bicolor,* and *Oryza sativa* Among Four Gene Regions

	Signal Sequence	Chy1 Region	Chy2 Region	Structural Region	χ^2	*P*	Significant Pairwise Comparisons
Polymorphic within *Zea*[a]							
Number of sites	3	0	3	17			
	45	27	45	210	2.5	NS	
Total fixed differences							
Changes	8	1	15	26			
Number of sites	39	27	36	183	12.45	<0.01	chy1 vs. chy2,** chy2 vs. SR**
Synonymous differences							
Changes	3	1	4	19			
Number of sites	9	7	9	40	1.45	NS	
Replacement differences							
Changes	5	0	11	7			
Number of sites	30	20	27	143	23.90	<0.001	chy1 vs. chy2,** chy2 vs. SR***
Insertions-deletions							
Changes	1	0	4	3			
Number of sites	13	9	12	61	8.25	<0.05	chy2 vs. SR*
Synonymous replacement							
Synonymous	3	1	4	19			
Replacement	5	0	11	7	10.05	<0.05	chy2 vs. SR**

NOTE.—The four gene regions were a signal sequence cleaved prior to forming the mature protein, two inhibitory loops (chy1 and chy2), and the remainder of the coding region (structural region). Results from comparisons with the *O. sativa2* sequence are shown; analyses with the *O. sativa1* sequence produced similar results. Number of sites refers to number of nucleic acids except for insertions-deletions, for which number of sites refers to number of amino acids. SR indicates structural region, or those parts of the coding sequence that are not part of the signal sequence, the chy1 region, or the chy2 region.

[a] Polymorphic-sites data are from all *Zea* taxa combined. Polymorphic sites in individual taxa were distributed similarly. The total number of sites differs from the number of sites used in the calculations of fixed differences because of insertions and deletions that differentiated *Zea* from the other taxa.

* $P < 0.05$ (Fisher's exact test).

** $P < 0.01$ (Fisher's exact test).

*** $P < 0.001$ (Fisher's exact test).

ever, relative-rate tests conducted on eight other nuclear genes revealed no evidence for an elevated rate of evolution in *Zea* relative to *S. bicolor*. Moreover, the majority of changes that differentiate *wip1* from *Zea* and *S. bicolor* are replacement changes that occurred during the evolution of *Zea*, as would be expected if selective forces were responsible for the significant rate heterogeneity. We also do not think that the elevated evolutionary rate detected in the *Zea* lineage is the result of a relaxation of selective constraint. A relaxation in selective constraint should be accompanied by high rates of intrataxon polymorphisms. However, polymorphisms in *wip1* are within the range of polymorphisms found in neutrally evolving loci from *Zea*. Moreover, the chy2 region of *wip1*, which was largely responsible for the significant elevation in evolutionary rates, actually had lower levels of polymorphism than the structural regions of the gene. Thus, it seems more likely that the accelerated evolutionary rate in *Zea* is due to changes in selective forces. Rapid divergence of defense genes is consistent with some theoretical models that predict rapid divergence of defense alleles among related evolutionary lineages (Haldane 1949; Clarke 1976) and has been detected in chitinase, which is involved in plant defense against fungal pathogens, among *Arabis* species (Bishop, Dean, and Mitchell-Olds 2000).

In contrast to the results from these intergeneric analyses, results from intraspecific analyses do not indicate that *wip1* has evolved in response to selection. The intraspecific and intergeneric tests may produce inconsistent results because the intraspecific tests for selection may have low statistical power (Wayne and Simonsen 1998). We propose two possible biological reasons for the inconsistency. One of these possibilities is that there have been temporal fluctuations in selective pressures that have acted on *wip1*. In particular, if *wip1* does evolve in response to selection imposed by herbivores and pathogens, then bouts of selection resulting from population outbreaks or shifts in the communities of herbivores and pathogens that attack *Zea* may have caused rapid evolutionary change. However, if the most recent bout of selection has been followed by a long period of selective neutrality, the intraspecific analyses will not detect evidence of selection, i.e., reduced diversity, because the signal has been lost through the accumulation of genetic diversity.

An alternative biological explanation for no evidence of selection being detected with the intraspecific tests is that *wip1* evolves in a manner that is not detected by these analyses (Bergelson et al. 2001). For example, when a new defense allele enters a population, there may be no effective counterdefenses in the parasite pop-

ulation, and thus that allele may confer a selective advantage and increase in frequency. However, as that new defense allele increases in frequency, parasite counterdefenses will be selectively favored, and by the time the new defense allele becomes common, parasite counterdefenses may also be common, negating the selective advantage that was initially associated with the defense allele. Once counterdefenses have evolved, the once-advantageous defense allele may be selectively neutral and gradually lost from a population. In other words, *wip1* alleles may be selectively favored only when they are uncommon and have been recently introduced into a population; i.e., *wip1* alleles experience novel allele advantage. Because novel allele advantage would not result in either fixation of new alleles or the maintenance of alleles via balancing selection, results from molecular tests of nonneutral evolution, which are generally designed to detect either positive or balancing selection, may be consistent with a neutral evolutionary history, even though selection is acting.

Evolutionary Rates Differ Between the Two Inhibitory Loops

In addition to finding that *wip1* has evolved faster in *Zea* than either *S. bicolor* or *T. dactyloides,* we found evidence that the two inhibitory loops of *wip1* have diverged at significantly different rates. Among 50 sequences from the four genera, including the duplicated genes in *O. sativa,* we detected only a single, synonymous, change in the chy1 inhibitory loop. The lack of divergence in the chy1 inhibitory loop is surprising given that the majority of previous investigations of PIs have revealed evidence of elevated rates of evolutionary change and hypervariability of amino acids in inhibitory loops (Hill and Hastie 1987; Laskowski et al. 1987; Creighton and Darby 1989; but see Beuning, Spriggs, and Christeller 1994). Similarly, the duplicated genes in *O. sativa* do not exhibit high rates of divergence in either of the inhibitory loops, even though these two genes differ at more than 36 sites, indicating that sufficient time has passed for differences to accumulate (data not shown). Taken together, these results show that rapid evolutionary divergence of inhibitor loops may not be as general a phenomenon among plant protease inhibitors as previously thought (Creighton and Darby 1989).

In contrast to the conserved chy1 region, the chy2 inhibitory loop has diverged rapidly. Although we cannot reject the possibility that chy2 has evolved neutrally, several aspects of the data are suggestive of a nonneutral evolutionary history. In particular, relative to other gene regions and other nuclear genes, the chy2 inhibitory loop has a higher ratio of nonconservative to conservative amino acid changes (data not shown), a higher ratio of fixed differences to polymorphic sites, an excess of fixed indels, and a significantly higher rate of divergence among *Zea*, *Sorghum*, *Tripsacum*, and *Oryza*, particularly at nonsynonymous sites (table 5). Many of these differences are not significantly different from expectations under a neutral model but are significantly different in the chy1 and chy2 regions (table 5). However, given that the chy2 region contains only 15 amino acids within *Zea*, and fewer than that in the other taxa, there was very little power to actually detect significant differences. In summary, the evidence for positive selection having acted on the chy2 region is equivocal, but the evidence for chy1 and chy2 regions having different selective histories is strong. An explanation for why these two inhibitory regions, which are predicted to have very different biochemical functions (Rohermeier and Lehel 1993), exhibit such different patterns of polymorphism and divergence will require more detailed analyses of these regions' biochemical and ecological functions.

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