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Multiallelic Recognition: Nonself-Dependent Dimerization of the bE and bW Homeodomain Proteins in Ustilago maydis

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Summary

In the plant pathogenic fungus Ustilago maydis, sexual and pathogenic development are controlled by the multiallelic *b* mating-type locus. The *b* locus encodes a pair of unrelated homeodomain proteins termed bE and bW, with allelic differences clustering in the N-terminal domains of both polypeptides. Only combinations of *bE* and *bW* of different allelic origin are active. We have investigated the underlying molecular mechanism for this intracellular self/nonself recognition phenomenon. By using the two-hybrid system, we were able to show that bE and bW dimerize only if they are derived from different alleles. Dimerization involves the N-terminal variable domains. Different point mutants of *bE2* were isolated that function in combination with *bW2*. The majority of such bE2 mutant polypeptides were also able to form heterodimers with bW2 in the two-hybrid system. Nonself-dependent dimerization of bE and bW was supported with a biochemical interaction assay with immobilized proteins. Our results suggest a model for self/nonself recognition in which variable cohesive contacts direct dimerization.

Introduction

The basidiomycete Ustilago maydis is a fungal pathogen causing smut disease of corn. After infection, plant tumors are induced in which the fungus proliferates and produces massive amounts of diploid spores (see Christensen, 1963; Banuett and Herskowitz, 1988; Banuett, 1992). Upon spore germination, nuclei undergo meiosis, and haploid sporidia are generated. The haploid cells grow yeast-like. They can be propagated on artificial media and are amenable to genetic and molecular analysis; however, they are unable to infect plants. The infectious form is generated by fusion of two compatible haploid cells. The resulting infectious dikaryon undergoes a dramatic morphological transition and assumes hyphal growth. Dikaryon formation can be visualized on complete medium to which charcoal has been added. Aerial hyphae of the dikaryon lead to a fuzzy appearance of the colonies (Fuz^+ phenotype). The filamentous dikaryon arises on artificial media but cannot continue to grow. It has to enter its host plant to complete the life cycle, which involves karyogamy, extensive cell proliferation, and spore formation (for reviews, see Christensen, 1963; Banuett and Herskowitz, 1988; Banuett, 1992).

Cell fusion, the dimorphic switch, and pathogenic development in U. maydis are controlled by two unlinked mating-type loci termed *a* and *b*. The *a* locus exists in the alleles *a1* and *a2*; the *b* locus is multiallelic. To form an infectious dikaryon, two haploid cells that carry different alleles in *a* and in *b* have to fuse (Rowell and DeVay, 1954; Rowell, 1955; Holliday, 1961; Puhalla, 1970; Day et al., 1971). The *a* locus controls the cell fusion event through a pheromone-based recognition system (Bölker et al., 1992; Spellig et al., 1994). Once the dikaryon is formed, the *a* locus continues to function in the maintenance of hyphal growth, presumably by autocrine activation of the pheromone response pathway (Banuett and Herskowitz, 1989; Bölker et al., 1992; Spellig et al., 1994).

In the dikaryon, the status at the multiallelic *b* locus determines further development (Holliday, 1961; Puhalla, 1968; Banuett and Herskowitz, 1989). In U. maydis, there exist at least 33 different *b* alleles in nature (Rowell and DeVay, 1954; Puhalla, 1968; J. E. DeVay, quoted by Wong and Wells, 1985). Pathogenic development is induced if the two nuclei in the dikaryon carry different *b* alleles; this pathway is blocked, however, if the two nuclei carry identical *b* alleles. This implies that there are at least 528 different *b* combinations that trigger pathogenic and sexual development, while 33 *b* allele combinations are inactive. It is one of the most fascinating tasks to elucidate how this process of intracellular recognition between self and nonself operates molecularly. This phenomenon is not unique to U. maydis but appears to be a general mechanism to control developmental switches such as induction of fruiting body formation or pathogenicity in several basidiomyceteous fungi (Kües et al., 1992; Specht et al., 1992; Bakkeren and Kronstad, 1993).

The *b* locus in U. maydis contains a pair of genes that are divergently transcribed. The two genes are termed *bE* and *bW*, and their predicted polypeptides comprise 473 and 644 amino acids, respectively (Schulz et al., 1990; Gillissen et al., 1992; see Experimental Procedures). The coding regions are separated by a 200 bp spacer. Each allele of the *b* locus consists of such a gene pair. In different *b* alleles, a region of about 1000 bp that contains the 5′ ends of both genes and the intergenic spacer shows a high degree of sequence variability, while the flanking sequences that encode the C-terminal domains of *bW* and *bE* show very little sequence variation. Determinants of allele specificity have been mapped to the variable domains of bE polypeptides (Dahl et al., 1991; Yee and Kronstad, 1993). While similar in organization, the products of the *bE* and *bW* genes are not related by amino acid sequence, except for a fungal homeodomain motif located in the constant portions immediately following the variable domains (Schulz et al., 1990; Gillissen et al., 1992). The finding that *b*-null mutants are unable to enter the sexual cycle excludes the possibility that it is the combination of identical *b* alleles that represses development. This has led to the suggestion that b polypeptides derived from different alleles form complexes that regulate develop-

ment (Schulz et al., 1990; Gillissen et al., 1992). The active b complex could either activate genes involved in pathogenicity (Gillissen et al., 1992) or act as a repressor of a repressor controlling pathogenicity genes (Banuett, 1991). As yet, no direct targets for the b polypeptides have been identified.

An analysis of mutants deleted for either *bE* or *bW* revealed that *b* locus function is seen only in those cases in which *bW* and *bE* genes derived from different alleles are combined. For a cross between two compatible haploids, this implies that there are usually two active *b* combinations that can be formed, raising the number of potentially active pairs of *b* genes to 1056. The finding that only certain combinations confer activity makes it likely that the respective bE and bW polypeptides interact physically and that this interaction is necessary for their regulatory function. As yet, it is unknown what determines the difference between an active and an inactive combination of b polypeptides. Basically, two scenarios have been proposed. In one, activity coincides with the ability of heterodimers to bind to specific target sequences; in the other, activity is determined at the level of heterodimer formation, which then targets the complex to the appropriate sites (see Kües and Casselton, 1992).

To investigate the mechanism of self/nonself recognition experimentally, we have employed the Saccharomyces cerevisiae two-hybrid system developed to detect protein–protein interactions (Fields and Song, 1989; Fields and Sternglanz, 1994). Our studies demonstrate that bE and bW form heterodimers only if they are derived from different alleles of the *b* locus. Dimerization occurs through the variable regions of the bE and bW polypeptides and does not require the homeodomain motifs.

Results

bE and bW Dimerize Only If They Are Derived from Different Alleles

To study the combinatorial interactions between bE and bW, we have used the two-hybrid system (Fields and Song, 1989). We fused the coding regions of *bW1*, *bW2*, *bE1*, and *bE2* either to the C-terminus of the GAL4 DNA-binding domain (GB) or to the N-terminus of the GAL4 activation domain (GA). Plasmid nomenclature identifies constructs by the length of the *b* open reading frame (ORF); e.g., pGB-$W2_{546}$ is a plasmid encoding a fusion protein in which the GAL4 DNA-binding domain is fused to the N-terminus of bW2 encompassing amino acids 1–546.

Control experiments (data not shown) demonstrated that bW carries a domain for transcriptional activation in yeast, while bE does not. This domain maps between amino acids 546 and 567. Since its removal did not affect bW function in U. maydis, we used bW_{546} in subsequent experiments where bW was fused to the DNA-binding domain of GAL4. To facilitate cloning steps, we also worked with a truncated version of bE (bE_{332}) that is functional in U. maydis (Schulz et al., 1990).

Different GB- and GA-fusion plasmids were transformed in S. cerevisiae strain Y153 in various combinations, and transformants were scored for *lacZ* activity (Figure 1B).

Remarkably, bE and bW proteins could interact only when they originated from different alleles of the *b* locus: both pGB-$E1_{473}$ combined with pW2_{632}-GA and pGB-$E2_{473}$ combined with pW1_{631}-GA could activate transcription of the *lacZ* reporter gene (Figure 1B). Activation was not seen, however, when pGB-$E2_{473}$ was combined with pW2_{632}-GA or when pGB-$E1_{473}$ was combined with pW1_{631}-GA (≤0.02 U, Figure 1B). The same result was obtained when bW was fused to the DNA-binding domain and when bE was fused with the DNA activation domain of GAL4 (Figure 1B). β-Galactosidase activity was undetectable when either plasmid was tested alone (Figure 1B).

Allele-Specific Interactions between bE and bW Involve the Variable Domains

To investigate which domains participate in heterodimer formation, we have generated C-terminal deletion derivatives of bE and bW that were fused to either the DNA-

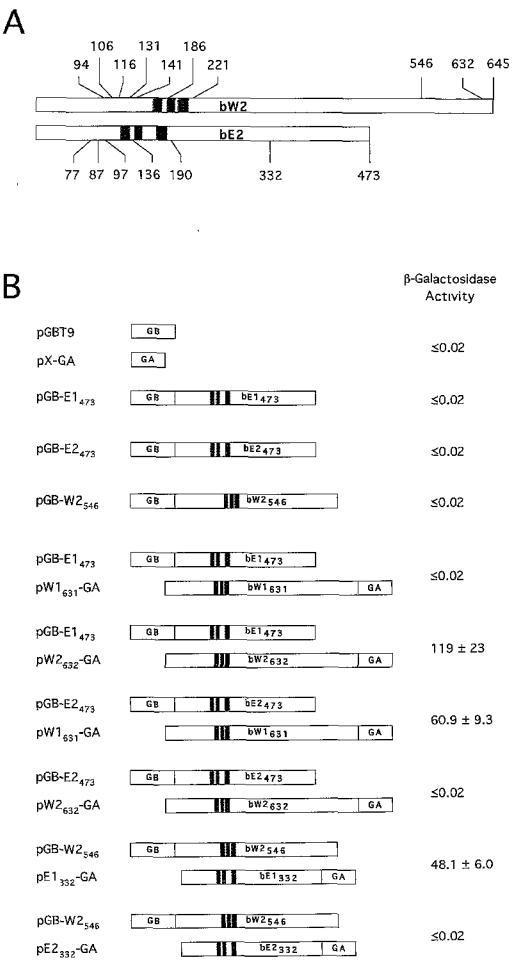

Figure 1. Allele-Specific Interactions between bE and bW

(A) Schematic diagram of the coding regions for bE and bW deletion endpoints. The shaded areas mark the three helices of the homeodomains. The coding region of bW1 comprises only 644 amino acids (Gillissen et al., 1992).

(B) Plasmids expressing the fusion proteins indicated schematically were tested alone or in the pairwise combination for their ability to activate the *lacZ* reporter gene in Y153. GB denotes the DNA-binding domain of GAL4; GA indicates the activation domain of GAL4. Numbers indicate β-galactosidase activity in units with standard deviation.

Table 1. The Variable Regions of bE and bW Are Sufficient for Allele-Specific Dimerization

bE–bW Combinations	Plasmid Expressing bE Fusion	Plasmid Expressing bW Fusion	β-Galactosidase Activity[a]
E1–W1	pGB-E1$_{136}$	pW1$_{186}$-GA	≤0.02
	pGB-E1$_{97}$	pW1$_{186}$-GA	≤0.02
	pGB-E1$_{87}$	pW1$_{186}$-GA	≤0.02
	pGB-E1$_{77}$	pW1$_{186}$-GA	≤0.02
E2–W2	pGB-E2$_{136}$	pW2$_{186}$-GA	≤0.02
	pGB-E2$_{97}$	pW2$_{186}$-GA	≤0.02
	pGB-E2$_{87}$	pW2$_{186}$-GA	≤0.02
	pGB-E2$_{77}$	pW2$_{186}$-GA	≤0.02
E2–W1	pGB-E2$_{136}$	pW1$_{186}$-GA	130.0 ± 51.1
	pGB-E2$_{97}$	pW1$_{186}$-GA	5.49 ± 4.10
	pGB-E2$_{87}$	pW1$_{186}$-GA	≤0.02
	pGB-E2$_{77}$	pW1$_{186}$-GA	≤0.02
E1–W2	pGB-E1$_{136}$	pW2$_{186}$-GA	193.3 ± 81.2
	pGB-E1$_{97}$	pW2$_{186}$-GA	101.0 ± 27.3
	pGB-E1$_{87}$	pW2$_{186}$-GA	9.5 ± 3.3
	pGB-E1$_{77}$	pW2$_{186}$-GA	8.0 ± 2.2
	pE1$_{136}$-GA	pGB-W2$_{186}$	74.4 ± 27.4
	pE1$_{136}$-GA	pGB-W2$_{141}$	215.4 ± 148.8
	pE1$_{136}$-GA	pGB-W2$_{131}$	78.8 ± 35.3
	pE1$_{136}$-GA	pGB-W2$_{116}$	8.61 ± 0.83
	pE1$_{136}$-GA	pGB-W2$_{106}$	8.18 ± 1.47
	pE1$_{136}$-GA	pGB-W2$_{94}$	4.06 ± 0.94
	pE1$_{77}$-GA	pGB-W2$_{94}$	19.9 ± 2.9

[a] β-Galactosidase activity was assayed in Y153 as described in Experimental Procedures; numbers indicate β-galactosidase activity in units ± SD.

binding or the activation domain of GAL4 (see Figure 1A and Experimental Procedures for details). Various plasmid combinations were assayed for their ability to activate the *lacZ* reporter gene system (Table 1). Full activation was seen when bE1$_{136}$ was combined with bW2$_{186}$ and when bE2$_{136}$ was combined with bW1$_{186}$. bE2$_{97}$ combined with bW1$_{186}$ displayed a 25-fold decrease in β-galactosidase activity when compared with the bE2$_{136}$-bW1$_{186}$ combination. bE2$_{87}$ and bE2$_{77}$ were inactive. For the reciprocal combination where truncations of bE1 were combined with bW2$_{186}$, a different picture emerged: a 10-fold decrease in β-galactosidase activity was observed between deletion endpoints 97 and 87; furthermore, bE1$_{77}$ was still active. All truncated bE1 and E2 proteins that were still active retained their allele specificity as determined in combination with bW1$_{186}$ and bW2$_{186}$, respectively (Table 1). bW2 derivatives retaining only parts of the variable domain up to deletion endpoint 131 displayed high levels of activity in combinations with bE1$_{136}$; shorter versions showed about 15-fold less activity (bW2$_{116}$, bW2$_{106}$, and bW2$_{94}$). To demonstrate that only parts of the variable region are sufficient for interaction, we also assayed a combination of bE1$_{77}$ and bW2$_{94}$. The 20 U of β-galactosidase activity measured indicated that such short peptides can still interact (Table 1). These results lead us to conclude that contacts between the variable domains of bE and bW are critical for allele-specific heterodimer formation. The presence of the homeodomains is not necessary for this interaction.

Isolation and Mapping of Point Mutations in *bE* That Render an Inactive *b* Gene Combination Active

The results presented thus far illustrate that active bE–bW combinations differ from inactive ones by their ability to form heterodimers. Is this the only criterion that distinguishes active and inactive polypeptide combinations in U. maydis? We had previously identified a *bE3* point mutant that was active for initiating pathogenic development when combined with *bW3* (F. Schauwecker and R. K., unpublished data). We now wanted to know whether similar mutations could be isolated in bE2 and whether such mutant proteins had acquired the ability to form heterodimers with bW2. We used misincorporating polymerase chain reaction (PCR) to mutagenize the variable domain of *bE2* encoding amino acids 12–223 in plasmid pUM-E2 (see Experimental Procedures for details). This autonomous high copy number plasmid contains the entire coding region of *bE2* and transforms U. maydis with high frequency (Tsukuda et al., 1988). The mutagenized plasmid population was introduced into the diploid strain FBD11-21 (*a1a2b2b2*). In this strain, pUM-E2 does not induce filamentous growth (Table 2), because no active bE–bW complexes can be formed. About 0.01% of the transformants obtained from the mutagenized plasmid pool showed filamentous growth after replica plating onto charcoal plates. Plasmids from such transformants (pUM-E2mut#) were reisolated and analyzed after transformation in Escherichia coli (see Experimental Procedures). In four mutants, single amino acid substitutions at different positions in the variable domain were observed (Table 2; Figure 2). In the other mutants, up to four amino acids in the region between amino acid 13 and amino acid 151 were altered. To verify that these mutant alleles turn on pathogenic development in U. maydis, stable transformants were generated in strain FBD11-21 (*a1a2b2b2*) (see Experimental

Table 2. bE2 Mutations Leading to Interaction with bW2: Screening in U. maydis

Plasmid in U. maydis	Phenotype in U. maydis[a] Fuz	Tum	Plasmid in S. cerevisiae	Two-Hybrid System[b] Interaction with $bW1_{186}$	$bW2_{186}$	Mutation
pUM-E2mut#22	+	+	pPE2-GAmut#22	107.7 ± 22.4	32.0 ± 6.0	H87L
pUM-E2mut#34	+	+	pPE2-GAmut#34	134.8 ± 6.4	0.20 ± 0.06	T94I
pUM-E2-mut#49	+	+	pPE2-GAmut#49	110.8 ± 17.1	4.49 ± 1.01	K70I
pUM-E2mut#37	+	+	pPE2-GAmut#37	122.5 ± 15.2	0.07 ± 0.03	A90V
pUM-E3mut#88	+	(−)	pPE2-GAmut#88	25.8 ± 6.5	≤0.02	A90V, H121R
pUM-E2mut#23	+	+	pPE2-GAmut#23	19.6 ± 1.3	≤0.02	P32L, A90V, P135L
pUM-E2mut#15	+	+	pPE2-GAmut#15	61.5 ± 12.6	18.0 ± 6.8	K74E, K100R, N148S
pUM-E2mut#66	+	+	pPE2-GAmut#66	87.4 ± 21.5	10.2 ± 1.2	K74E, E92G, T151S
pUM-E3mut#45	+	+	pPE2-GAmut#45	126.8 ± 14.9	7.38 ± 1.57	V13L, F23V, H60Q, K100E
pUM-E2	−	−	$pPE2_{332}$-GA	84.9 ± 46.1	≤0.02	none
			$pE2_{332}$-GA	24.2 ± 10.8	≤0.02	none

[a] Plasmids were stably introduced in U. maydis strain FBD11-21 (*a1a2b2b2*), and the Fuz phenotype was determined by streaking colonies on PD–charcoal plates. Plus indicates a Fuz$^+$ phenotype; minus indicates a Fuz$^-$ phenotype. To determine the Tum phenotype, pure cultures of at least three stable transformants were assayed in planta; plus indicates tumor induction, minus in parentheses indicates anthocyanin synthesis but no tumor formation, and minus indicates that no disease symptoms could be detected.

[b] The yeast strain Y153 was cotransformed with plasmids pPE2-GAmut# and pGB-$W1_{186}$ or pGB-$W2_{186}$, respectively. β-Galactosidase activity was assayed as described in Experimental Procedures and is indicated in units ± SD. For comparison with β-galactosidase activity in Figure 1 and Table 1, where a less sensitive two-hybrid system was used, values there have to be multiplied by a factor of about 4.

Procedures). For each mutant allele, at least three independent transformants were analyzed. In all cases except pUM-E2mut#88, more than 50% of the infected plants developed full disease symptoms (Table 2). pUM-E2mut#88 transformants were able to induce anthocyanin synthesis but failed to induce tumors. This illustrates that all these *bE2mut* alleles were recognized as nonself (*non-bE2*) in a *b2* strain. Next, we asked whether the novel specificities of these *non-bE2* alleles corresponded to those of reference alleles. For this experiment, RK2175 (*a1Δb*), which carries a deletion of the *b1* locus, was transformed with plasmids pUM-E2mut#22, pUM-E2mut#34, pUM-E2mut#37, and pUM-E2mut#45 (see Table 2). Three transformants each were crossed with *a2* strains carrying *b1* (FB6a), *b3* (RK32), and *b4* (RK1320) and with *a2* strains from the American Type Culture Collection (ATCC) carrying the *b* alleles designated *bA*, *bD*, *bE*, *bF*, *bG*, *bH*, *bL*, *bN*, *bO*, and *bP*. All transformants gave a Fuz$^+$ reaction with all tester strains (data not shown). This indicates that the mutations in pUM-E2mut#22, pUM-E2mut#34, pUM-E2mut#37, and pUM-E2mut#45 did not create alleles of these known specificities.

Do these bE2 mutant polypeptides that are active in combination with bW2 form heterodimers with bW2? To investigate this question, all *bE2mut* genes were analyzed in the two-hybrid system. The mutated regions of several plasmids (see Table 2) were used to replace the *bE2* wild-type fragment in $pE2_{332}$-GA (see Experimental Procedures). When tested in combination with pGB-$W2_{186}$, only one of the plasmids tested (pE2-GAmut#22) was able to activate *lacZ* in yeast strain SFY526 (data not shown). To enhance sensitivity, the *ADH* promoter in the pE2-GAmut# constructs was replaced by a stronger version of the same promoter originating from pGAD-GH (compare $pPE2_{332}$-GA and $pE2_{332}$-GA in Table 2). Exchanging the promoter for the stronger version and using strain Y153 increased sensitivity about 8-fold. The resulting plasmids pPE2-GAmut#15, pPE2-GAmut#22, pPE2-GAmut#23, pPE2-GAmut#34, pPE2-GAmut#37, pPE2-GAmut#45, pPE2-GAmut#49, pPE2-GAmut#66, and pPE2-GAmut#88 were combined with pGB-$W2_{186}$ in strain Y153 and assayed for β-galactosidase activity (Table 2). Except for two mutants, all plasmids induced detectable β-galactosidase activity ranging between 0.07 and 32 U. When combined with pGB-$W1_{186}$, all plasmids directed high levels of β-galactosidase synthesis (Table 2), indicating that interactions with bW1 were not grossly affected by the mutations. These data show that most mutant alleles of *bE2* that were selected in U. maydis by their ability to induce filamentous growth and tumor formation in a *b2* genetic background encode proteins that can form heterodimers with bW2 when assayed in the yeast two-hybrid system. The two mutant alleles *bE2mut23* and *bE2mut88* selected for filamentous growth

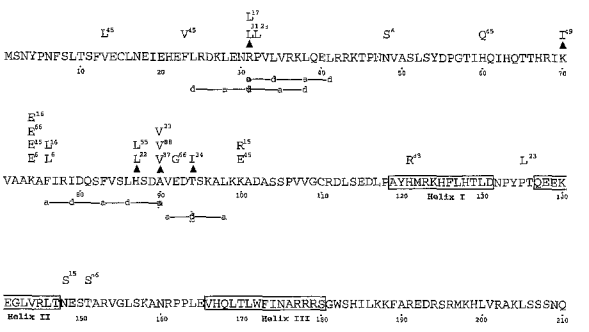

Figure 2. N-Terminal Amino Acid Sequence of bE2 and Location of Mutations That Alter Its Specificity

Triangles denote positions where the single amino acid exchanges indicated were found. Superscripted numbers denote the numbers of the respective plasmids in which the mutation(s) was isolated (see Tables 2 and 3). The putative helices of the homeodomain are boxed. Several short stretches that could fold in amphipathic helices are indicated by small letters, with a and d denoting hydrophobic amino acids at the critical positions 1 and 4 of the heptad repeat (see Hurst, 1994).

Table 3. bE2 Mutations Leading to Interaction with bW2: Screening in S. cerevisiae

	Two-Hybrid System[a] Interaction with			Phenotype in U. maydis[b]		
Plasmid in S. cerevisiae	$bW1_{186}$	$bW2_{186}$	Plasmid in U. maydis	Fuz	Tum	Mutation(s)
pPE2-GAmut#6	83.5 ± 5.6	55.7 ± 4.8	pUM-E2Ymut#6	+	+	N47S, K74E, F76L
pPE2-GAmut#16	104.0 ± 14.3	23.3 ± 6.5	pUM-E2Ymut#16	+	+	K74E, F76L
pPE2-GAmut#31	16.7 ± 8.9	38.5 ± 8.0	pUM-E2Ymut#31	+	+	R31L
pPE2-GAmut#55	99.6 ± 54.6	31.2 ± 4.4	pUM-E2Ymut#55	+	+	H87L, S218Y
pPE2-GAmut#17	15.2 ± 1.0	24.8 ± 4.9	pUM-E2Ymut#17	+	ND	R31L (L130L)
pPE2-GAmutRH	0.18 ± 0.08	132.5 ± 29.3	pUM-E2mutRH	+	ND	R31L, H87L
pPE2-GAmutRT	1.37 ± 0.28	107.7 ± 23.7	pUM-E2mutRT	+	ND	R31L, T94I
$pPE2_{332}$-GA	84.9 ± 46.1	≤0.02	pUM-E2	−	−	none
$pE2_{332}$-GA	24.2 ± 10.8	≤0.02				

[a] See footnote b in Table 2.
[b] See footnote a in Table 2.

in U. maydis did not lead to detectable interactions with bW2 in the two-hybrid system. This indicates that very small differences in affinity are sufficient to provide for function in U. maydis. Interestingly, pUM-E2mut#88 transformants were greatly attenuated in pathogenicity.

Mutants Selected for Heterodimer Formation in S. Cerevisiae Confer Activity in U. maydis

In a converse approach to the one described in the preceding section, we isolated mutants on the basis of interaction in the two-hybrid system and then assayed for function in U. maydis. After mutagenesis of the variable region in $pE2_{332}$-GA, the plasmid pool was introduced in yeast strain SFY526 harboring pGB-$W2_{186}$, and colonies expressing *lacZ* were isolated (see Experimental Procedures). From such strains, the respective pE2-GAmut# plasmids were isolated. By sequence analysis of five mutants, two with a single amino acid exchange, two with two amino acid exchanges, and one with three amino acid substitutions were identified (Table 3; Figure 2). Interestingly, the H87L mutation had also been identified in the U. maydis screen (see Table 2). To quantitate data and to allow comparison with data in Table 2, the mutations were introduced in $pPE2_{332}$-GA to generate plasmids pPE2-GAmut#6, pPE2-GAmut#16, pPE2-GAmut#17, pPE2-GAmut#31, and pPE2-GAmut#55. As expected, all mutant proteins were active in combination with bW2 in the two-hybrid system; in general, values of β-galactosidase activity were significantly higher than for the mutants that were selected for filament formation in the U. maydis screen (Table 3). This can be explained, because a less sensitive two-hybrid system was used for selection (pE2-GAmut# in SFY526), and weaker interactions would have gone unnoticed.

For an analysis in U. maydis, mutations were introduced into pUM-E2 (see Experimental Procedures for details). The resulting plasmids pUM-E2Ymut#6, pUM-E2Ymut#16, pUM-E2Ymut#17, pUM-E2Ymut#31, and pUM-E2Ymut#55 were used to generate stable transformants of strain FBD11-21 (*a1a2b2b2*). Three independent transformants each were used for pathogenicity tests. In all cases, tumors were induced; i.e., the mutant alleles were active in the *b2* genetic background (Table 3). This result demonstrates that the ability of bE2mut polypeptides to form heterodimers with bW2 in yeast correlates with formation of an active *b* gene complex in U. maydis.

Point Mutations Can Act Synergistically

To assess the contribution of different amino acid substitutions for heterodimerization, we created two alleles that each combine two of the single amino acid substitutions. These plasmids, pPE2-GAmutRH (R31L and H87L) and pPE2-GAmutRT (R31L and T94I), were assayed in the two-hybrid system. In combination with $bW2_{186}$, both constructs showed levels of activity that were significantly higher than those observed for the original mutations (Tables 2 and 3). Surprisingly, both mutants showed very low β-galactosidase expression in combination with $bW1_{186}$ (Table 3). When recloned into the U. maydis vector and introduced into U. maydis strains FBD11-21 (*a1a2b2b2*) and FBD12-3 (*a1a2b1b1*), respectively, pUM-E2mutRH and pUM-E2mutRT induced a Fuz^+ phenotype that indicates function (Table 3).

In Vitro Interactions between bE and bW Proteins

The allele-specific interactions between bE and bW observed in the two-hybrid system were confirmed by an in vitro protein interaction assay. His-tagged bE proteins His-$bE1_{190}$, His-$bE2_{190}$, and the bE2 mutant derivatives His-$bE2T94I_{190}$ and His-$bE2H87L_{190}$ were synthesized in E. coli (see Experimental Procedures) and were immobilized on Ni^{2+}–Sepharose beads. The bound proteins were then incubated with [^{35}S]methionine-labeled bW_{221} proteins that had been generated by in vitro transcription and translation (Figure 3C). After the beads had been washed extensively with a buffer containing 0.5 M NaCl, proteins were eluted with imidazole, separated on an SDS gel, and detected by autoradiography (for details, see Experimental Procedures). As can be seen in Figure 3A, $bW2_{221}$ binds to $bE1_{190}$, and $bW1_{221}$ binds to $bE2_{190}$; combinations where both partners were derived from the same allele showed only a weak signal that is comparable in intensity with the control, where binding was assayed with Ni^{2+}–Sepharose beads without immobilized bE protein. The mutant bE2 proteins His-$bE2T94I_{190}$ and His-$bE2H87L_{190}$ behaved like

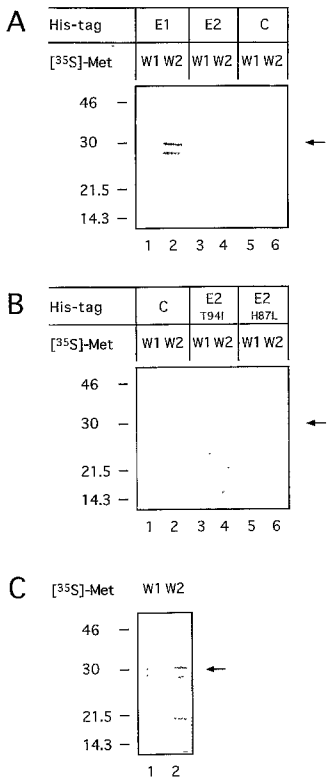

Figure 3. Interaction between bE and bW Proteins In Vitro

Isolated, His-tagged bE proteins His-bE1$_{190}$, His-bE2$_{190}$, and the bE2 derivatives His-bE2T94I$_{190}$ and His-bE2H87L$_{190}$ carrying the amino acid substitutions T94I and H87L, respectively (see Tables 2 and 3), were bound to Ni^{2+}–Sepharose beads and tested for binding of [^{35}S]methionine-labeled bW1$_{221}$ and bW2$_{221}$ proteins translated in vitro as described in Experimental Procedures. As a control, E. coli whole-cell extract prepared from BL21::DE3[pET15b] was bound to Ni^{2+}–Sepharose. After the beads had been washed with 0.5 M NaCl, bound proteins were eluted with imidazole and analyzed by 12% SDS–PAGE. Autoradiographs are shown. Numbers (left) mark positions of protein standards (in kilodaltons); the arrow indicates in vitro translated bW$_{221}$ protein.

(A) His-bE1$_{190}$ (lanes 1 and 2), His-bE2$_{190}$ (lanes 3 and 4), and control beads (lanes 5 and 6) were incubated with bW1$_{221}$ (lanes 1, 3, and 5) or bW2$_{221}$ (lanes 2, 4, and 6).

(B) bE2 proteins His-bE2T94I$_{190}$ (lanes 3 and 4) and His-bE2H87L$_{190}$ (lanes 5 and 6) and control beads (lanes 1 and 2) were incubated with bW1$_{221}$ or bW2$_{221}$ as in (A).

(C) The products of the in vitro translation reaction bW1$_{221}$ (lane 1) and bW2$_{221}$ (lane 2) used for the binding assay. The smaller translation products produced result from a second start codon located either 54 bp (bW1) or 57 bp (bW2) downstream of the first initiation codon used. As can be seen in (A) and (B), these shorter bW proteins also interact with bE$_{190}$ in an allele-specific manner.

the wild-type protein with respect to their interaction with bW1$_{221}$ (Figure 3B). In contrast with His-bE2$_{190}$, we found, however, that His-bE2H87L$_{190}$ was also able to bind bW2$_{221}$. For the combination of His-bE2T94I$_{190}$ with bW2$_{221}$, we detected a weak binding barely above background, which is consistent with results from the two-hybrid system.

Discussion

We have identified the variable regions of the bE and bW polypeptides as protein–protein interaction domains. Dimerization of bE and bW is allele specific and appears to be sufficient for function: all *bE2* mutant alleles that were selected for heterodimer formation with bW2 in yeast were functional in a U. maydis *b2* strain. Conversely, all but two mutants that were selected for activity in U. maydis lead to heterodimer formation in yeast. The tight connection between interaction in yeast and function in U. maydis is highlighted by the fact that the same single amino acid substitution (H87L) in the variable domain was identified in both screens.

Heterodimerization of DNA-binding proteins is a common mechanism to create composite transcription factors with novel specificities. In general, two distinct molecular mechanisms can operate. Both partners can contribute for DNA binding, and this allows the recognition of a new target. Alternatively, only one partner may provide a DNA-binding domain, while the other contributes a regulatory domain or a nuclear localization domain. It is presently unclear which of these mechanisms applies for the bE–bW heterodimers in U. maydis.

Using the two-hybrid system and a biochemical assay, we have demonstrated that allele-specific interactions involve the variable domains of the bW and bE proteins and do not require the homeodomains. N-terminal fragments of the variable domains exhibit different levels of interaction or lose the ability to interact, depending on length and allelic combination (Table 1). These results indicate that there exists more than one contact site and that these sites are located at different positions in different allelic combinations. In our biochemical assay, allele-specific binding was maintained under high salt conditions (0.5 M NaCl), suggesting that hydrophobic interactions are involved. In all combinations where bE and bW polypeptides were derived from the same allele, we were unable to detect a physical interaction.

How much interaction does Ustilago need to distinguish between self and nonself? Mutant alleles selected for function in U. maydis make it obvious that very weak interactions corresponding to 0.07 U of β-galactosidase activity are clearly sufficient to trigger filamentous growth and pathogenicity. For two of the mutant alleles, *bE2mut23* and *bE2mut88*, no measurable interaction could be detected in the two-hybrid system. Of these, the mutant allele *bE2-mut23* induces both filamentous growth and tumor development, while *bE2mut88* induced filamentous growth but caused only attenuated disease symptoms. These data indicate that even interactions that cannot be detected in the two-hybrid system are sufficient for U. maydis to classify these alleles as nonself. In one case, these differences are sufficient to trigger the entire developmental program, while in the other case, the interactions are so weak that they trigger filamentous growth, but further development is stalled at an intermediate point. Thus, U. maydis can discriminate between self and nonself at the level of protein dimerization.

When extended to all 33 naturally occurring *b* alleles, this would mean in molecular terms that a given bE or bW protein can form heterodimers with 32 different partners, but interaction between bE and bW originating from the same allele is specifically excluded. Within the variable

domain, there must be determinants for promiscuous interactions with many different partners, and there must be specific determinants that prevent interaction between bE and bW from the same allele. The mutants generated in this study are all affected in self recognition but not in nonself recognition. This suggests that residues crucial for noninteraction are altered in the mutants.

Noteworthy is that substitutions do not cluster in a specific region of the variable domain, and only characteristic types of amino acid substitutions lead to activity. In particular, all five single point mutations isolated increase hydrophobicity. In all but two mutants carrying more than one mutation, at least one substitution leads to an increase in hydrophobicity. Interestingly, both exceptions (pUM-E2#15 and pUM-E2#66; see Table 2) have the K74E mutation in common, which results in a change of charge. It thus appears that the ability of bE and bW to interact is governed mainly by hydrophobic interactions, polar interactions, or both. For proteins that engage in combinatorial interactions, this very often involves hydrophobic contacts through coiled-coil motifs (O'Shea et al., 1992; Hurst, 1994). These motifs exist in the large class of bZIP proteins and have also been suggested to constitute the dimerization domains of the a1/α2 repressor of yeast (Ho et al., 1994). A reinspection of the amino acid sequences of bE and bW polypeptides does not support the existence of extensive coiled-coil motifs. However, in bE, multiple short elements can be found that might fit into a coiled-coil protein folding pattern. Interestingly, some of the mutations leading to activity enlarge such putative 3,4-hydrophobic heptad sequences (Figure 2).

Since we do not have structural information on the variable domains of bE and bW, we would like to propose a schematic model that accommodates the data on the mutants and previously published data on chimeric alleles (Dahl et al., 1991; Yee and Kronstad, 1993). We assume that bE and bW carry multiple exposed hydrophobic or polar residues that can make cohesive contacts between bE and bW. As has been proposed for the preferential heterodimerization of Fos and Jun, polar residues of like charges prevent homodimerization, and those of opposite charge allow heterodimerization (Glover and Harrison, 1995). By a similar mechanism, a specific array of residues of like charge could prevent dimerization in combinations of bE and bW derived from the same allele (Figure 4A). When bE and bW polypeptides from different alleles are combined (Figure 4B), dimerization takes place through polar or hydrophobic residues. Note that the interacting pairs of amino acids do not need to be the same ones in different combinations of bE and bW polypeptides. This scenario also predicts that in a cross of two haploid strains, both of the two possible bE–bW combinations need not be productive, an assertion for which there exists genetic evidence (B. Gillissen and R. K., unpublished data).

Mutations that allow hydrophobic or polar interactions, such as the ones described in Tables 2 and 3 for the various *bE2mut* alleles, lead to an interaction between bE2mut and bW2 (Figure 4C, right). The strength of the interaction may well depend on the number of appropriate changes introduced and may explain why some of the combinations that were selected for function in U. maydis showed only very weak interaction. The synergistic effects observed when combining two single mutations lend further support to such a scheme. Our deletion data suggest that several cohesive contacts contribute to dimerization. Mutations in bE2 leading to an interaction with bW2 do not necessarily interfere with interactions with bW1, as was shown for most of the mutants (Tables 2 and 3). Certain bE2 mutations, however (such as R31L), can lower interactions with bW1 (Figure 4C, left). This indicates that the same residue that stabilizes interaction between bE2mut and bW2 can destabilize the bE2mut–bW1 interaction (see Figure 4C).

In the variable domain of bE2, the insertion of three amino acids at position 74 leads to an allele that is nonfunctional with bW1 (Schulz et al., 1990). The insertion could now be interpreted to result in displacement of all possible cohesive interactions beyond the insertion point. Alterna-

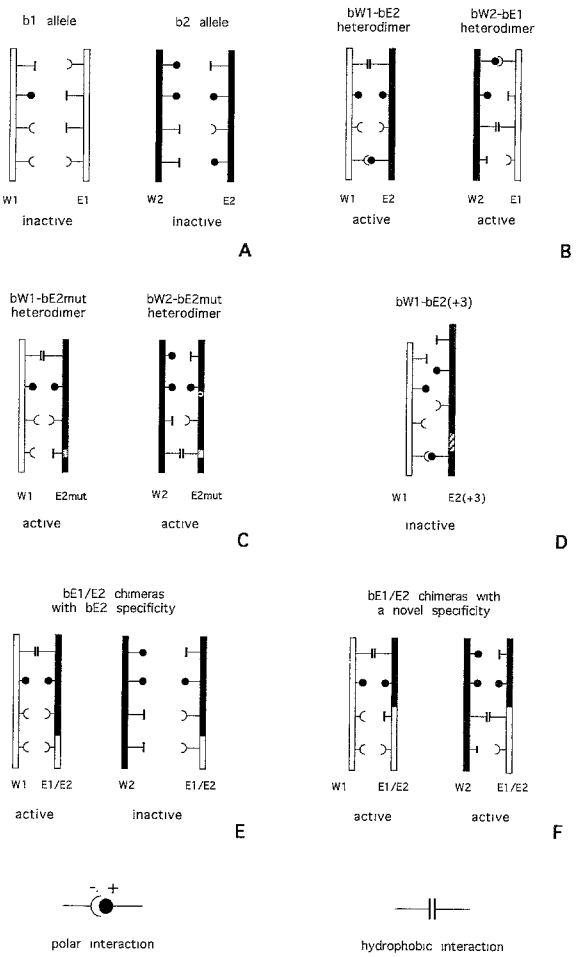

Figure 4. Model for Allele-Specific Dimerization

The model depicts schematically how the interfaces of the variable bE and bW domains come together when they are appropriately combined. Dimerization requires a complementary surface in at least one position; contacts could occur through hydrophobic residues or charged residues as depicted below. The shaded areas in (C) denote a point mutation; the hatched area in (D) indicates an insertion of three amino acids. All examples are referred to and explained in the Discussion.

tively, the insertion might prevent juxtaposition of adjacent homeodomains (Figure 4D). Interestingly, we have also found a naturally occurring *bW* allele (*bW5*) that has a two amino acid deletion in the variable domain. In crosses with several *bE* alleles, this allele was nonfunctional (M. Dahl and J. K., unpublished data).

In *bE*, specificity determinants have previously been mapped with the help of chimeric alleles (Dahl et al., 1991; Yee and Kronstad, 1993). Specificity regions were defined as those regions within which recombination created alleles with a specificity of neither parent (Yee and Kronstad, 1993). These domains were shown to reside at different positions within distinct *bE* alleles, which is consistent with our deletion analysis. How do these domains translate to critical contacts for specific interaction or noninteraction? We have depicted two prototype chimeras in our model (Figure 4E and 4F). The chimeric bE1/bE2 polypeptide shown in Figure 4E behaves like bE2, because all critical contacts are provided by the bE2 portion; i.e., this allele can interact with bW1 and cannot interact with bW2. The bE1/bE2 chimera with a new specificity (Figure 4F) can interact with bW1 as well as with bW2. According to our model, this novel specificity is generated because the fusion point is located between the critical cohesive residues in bE1 and bE2 that interact with bW (Figure 4F).

How many such cohesive contacts are required to accommodate the number of naturally occurring alleles? Let us assume that a single interaction is sufficient for dimerization: with four contact sites and the possibility that each residue can be either hydrophobic or polar (positively or negatively charged), the chances that two randomly generated patterns in bE and bW will interact is about 80%. If there are pairs of genes, however, the probability that at least one active combination occurs goes up to 96%. Thus, the simplistic model in Figure 4 that assumes only four residues interacting is able to generate enough variability to accommodate already 25 different *b* alleles that have been isolated from nature. Considering that it appears to be easy to alter the specificity of a given allele without generating already known specificities, the number of 33 alleles found in nature appears rather low. Does this indicate that for most of the mutant alleles, corresponding noninteracting bW proteins cannot be isolated? A complementary analysis of *bW* mutants and the elucidation of the structure of the interacting domains is expected to provide fundamental insight into this delicate mechanism of protein–protein recognition.

Experimental Procedures

Strains and Growth Conditions

Cloning in E. coli was done in DH5α (Bethesda Research Laboratories). For protein expression, E. coli BL21::DE3 (Studier et al., 1990) was used. Yeast strains used were SFY526 (*MATa, ura3-52, his3-D200, ade2-101, lys2-801, trp1-901, leu2-3,112, can*^{r}, *gal4-542, gal80-538, URA3::GAL1–lacZ*) (Bartel et al., 1993) and Y153 (*MATa, leu2-3,112, ura3-52, trp1-901, his3-D200, ade2-101, gal4D, gal80D, URA3::GAL–lacZ, LYS2::GAL-HIS3*) (Durfee et al., 1993). This strain harbors an additional ectopic copy of a *GAL1–lacZ* fusion. U. maydis strains were as follows: FBD11-21 (*a1a2b2b2*), FBD12-3 (*a1a2b1b1*), FB6a (*a2b1*), and RK2175 (*a1Δb*), which have been described (Banuett and Herskowitz, 1989; Schulz et al., 1990; Gillissen et al., 1992), and RK32 (*a2b3*). RK1320 (*a2b4*) was isolated as meiotic segregant from a cross of RK32 and RK138 (*a1b4*) (B. Schulz and R. K., unpublished data). The following strains of the ATCC were used: *a2bA* (ATCC 22899), *a2bD* (ATCC 22901), *a2bE* (ATCC 22902), *a2bF* (ATCC 22903), *a2bG* (ATCC 22904), *a2bH* (ATCC 22905), *a2bL* (ATCC 22909), *a2bN* (ATCC 22911), *a2bO* (ATCC 22912), and *a2bP* (ATCC 22913). U. maydis and S. cerevisiae were grown as described (Bölker et al., 1992; Sherman et al., 1986).

Transformation Procedures

Transformation of U. maydis was performed as described by Schulz et al. (1990) and Bölker et al. (1992). Transformation of S. cerevisiae was done following the protocol of Schiestl and Gietz (1989).

Pathogenicity Test

The pathogenicity test was described in Gillissen et al. (1992). Pathogenic strains induced anthocyanin biosynthesis and developed tumors on corn plants after 5–8 days (Tum$^+$ phenotype).

β-Galactosidase Activity of Yeast Transformants

Colony Assay

Transformants were lifted on nitrocellulose filters (Schleicher and Schuell Protran BA85/20), transferred to SSX plates (Chien et al., 1991), and incubated for 48 hr at 28°C. Filters were frozen in liquid nitrogen and incubated for 48 hr at 28°C on filter paper (Schleicher and Schuell 595) soaked with 1.5 ml of Z-buffer (Miller, 1972).

Quantification of β-Galactosidase Activity

Cells were grown in minimal medium containing raffinose to an OD_{600} of 0.8–1.2, and 500 μl of culture was used for measuring β-galactosidase activity, as described by Miller (1972). For each quantitation, ten independent transformants were tested in duplicate.

DNA Procedures

Isolation of U. maydis and S. cerevisiae DNA followed the protocols of Hoffman and Winston (1987) and Robzyk and Kassir (1992), respectively. DNA sequencing was done with a Sequenase Kit (United States Biochemical Corporation). PCR amplification reactions were according to the protocol of Innis and Gelfand (1990). Primers are indicated where the respective plasmids are described. All PCR-generated plasmid portions were sequenced. All other DNA manipulations followed standard procedures as described by Sambrook et al. (1989).

Plasmids and Plasmid Constructions

In the course of this study, we have found that the putative ORF for *bW* that had been proposed to start with amino acids MLPPL on the basis of the function of deletion derivatives (Gillissen et al., 1992) is likely to begin at an upstream in-frame ATG that would extend the ORFs of bW1 and bW2 by 18 or 19 amino acids, respectively (J. K., unpublished data). In our constructs containing *bW1* and *bW2*, we now refer to this ATG as the start codon.

pTZ18R, pT7T318U, pSL1180 (Pharmacia), pBluescript KS(+) (Stratagene), pSP72 (Promega), and pET15b (Novagen) were used for cloning DNA fragments. pHB40P is a pET3a (Studier et al., 1990) derivative with a modified linker (provided by A. Vershon, via A. Klippel at the University of California, San Francisco). pCM54 was described earlier (Tsukuda at al., 1988). In pGBT9 and pGAD424, either the GAL4 DNA-binding domain or the GAL4 activation domain is expressed from the *ADH1* promoter, followed by a polylinker, stop codons, and a terminator, respectively (Bartel et al., 1993). pGAD-GH is similar to pGAD424, but carries a larger *ADH1* promoter fragment that leads to higher expression values (G. Hannon, personal communication). pX-GA contains the nuclear localization signal (NLS) of pGAD2F (Chien et al., 1991), followed by a linker (5′-GAATTCCATATGGCCCG-GGTGTCGACGGGTACC-3′) with unique sites for EcoRI, NdeI, SmaI, SalI, and KpnI, and followed by the GAL4 activation domain. Cloning into the linker leads to fusion proteins with the NLS at the N-terminus and the GAL4 activation domain at the C-terminus. pPX-GA is a derivative with the stronger *ADH* promoter from pGAD-GH.

Plasmids pJBbE1 and pJBbE2 contain intron-free *bE1* and *bE2* genes cloned as NdeI–BamHI fragments into pHB40P. The NdeI site is located at the ATG; the BamHI site is located 83 bp (*bE1*) or 48 bp (*bE2*) downstream of the stop codon (Bergemann, 1993).

pW1-PXc codes for an intron-free copy (Gillissen et al., 1992) of *bW1*, cloned as a PstI–XbaI fragment in pSP72.

pBScW2 contains an intron-free XbaI–HaeII fragment harboring bW2 (Gillissen et al., 1992) cloned into pBluescript KS.

In pGB-E1473 and pGB-E2473, NdeI–BamHI fragments from pJBbE1 and pJBbE2 were blunted and inserted into the SmaI site of pGBT9.

In $pE1_{332}$-GA and $pE2_{332}$-GA, NdeI–SalI fragments from pJBbE1 and pJBbE2 were cloned into the respective sites of pX-GA. $pPE2_{332}$-GA is similar to $pE2_{332}$-GA, but carries the stronger promoter from pGAD-GH.

For plasmids pGB-$E1_{136}$, pGB-$E1_{97}$, pGB-$E1_{87}$, pGB-$E1_{77}$, pGB-$E2_{136}$, pGB-$E2_{97}$, pGB-$E2_{87}$, and pGB-$E2_{77}$, fragments of different length coding for the respective deletion proteins were generated by PCR using pJBbE1 and pJBbE2 as templates. Primers introduced EcoRI and NdeI sites at the 5′ end (ATG) and KpnI and BamHI sites at the 3′ end. Fragments were cleaved with EcoRI and BamHI and cloned into the respective sites of pGBT9.

Plasmids pGB-$W1_{186}$, pGB-$W2_{186}$, pGB-$W2_{141}$, pGB-$W2_{131}$, pGB-$W2_{116}$, pGB-$W2_{106}$, and pGB-$W2_{94}$ were constructed as described for the pGB-E deletions, but primers corresponding to the bW sequences and different templates pW1-PXc or pUb2-2 (Schulz et al., 1990) were used.

pE1-GA, pE2-GA, and pW2-GA deletion derivatives were constructed by excising NdeI–KpnI fragments from the respective pGB fusion plasmids and inserting them into the respective sites of pX-GA.

In $pGBW2_{546}$, a BglII–FspI fragment of pBScW2 coding for amino acids 4–546 was cloned between the BglII and SalI (blunt) sites of pGB-$W2_{186}$ to generate the bW2 coding region from amino acid 1 to amino acid 546.

In $pW1_{631}$-GA, a NdeI–BglII fragment from pGB-$W1_{186}$ coding for amino acids 1–69 and a BglII–NdeI (blunt) fragment from pW1-PXc (amino acids 70–631) were introduced into pX-GA digested with NdeI and SmaI.

In $pW2_{632}$-GA, a NdeI–BglII fragment of pGB-$W2_{186}$ coding for amino acids 1–4 and a BglII–NdeI (blunt) fragment from pBScW2 encoding amino acids 5–632 were introduced into pX-GA digested with NdeI and SmaI.

pUM-E2 is a pCM54 derivative harboring a 2.4 kb EcoRI fragment with *bE2* isolated from pUb2-4 (Schulz et al., 1990) with singular XbaI and HindIII sites within the coding region of *bE2*.

For $pTRbW1_{221}$ and $pTRbW2_{221}$, fragments encompassing either the *bW1* or *bW2* coding region from amino acid 1 to amino acid 221 were generated by PCR of pW1-PXc or pBScW2 introducing NdeI and BamHI sites. Resulting fragments were digested with NdeI and BamHI and cloned into the respective sites of pHB40P.

For $pTRbE1_{190}$ and $pTRbE2_{190}$, construction was as described for $pTRbW1_{221}$ and $pTRbW2_{221}$, except that primers corresponding to the bE sequences and different templates (pJBbE1 or pJBbE2) were used, and fragments were cloned into pET15b.

For $pETbE2_{190}$T and $pETbE2_{190}$H, a 260 bp SnaBI–XhoI fragment in $pTRbE2_{190}$ was exchanged with the respective fragments from pPE2GAmut#34 or pPE2GAmut#22, respectively.

Detailed descriptions of plasmid constructs can be obtained on request from J. K.

Mutagenesis of the Variable Domain of *bE2*

The region between amino acids 1 and 399 of *bE2* was amplified under conditions of misincorporation as described by Spee et al. (1993) using primers 5′-GGTTTAGTTTTGCGCGCTGGAT-3′ and 5′-GGAATTCCATATCTCTAACTACCCGAACTTT-3′ and pUM-E2 as template. For the mutant screen in U. maydis, amplified fragments were cleaved with HindIII and XbaI and used to replace the respective wild-type fragment (amino acids 12–223) in pUM-E2 to yield pUM-E2mut plasmids. Plasmid pools were introduced in U. maydis FBD11-21. Colonies were replica plated on charcoal–PD plates. Colonies with a Fuz^+ phenotype were isolated and purified on selective medium. DNA was isolated, and plasmids were recovered after transformation of E. coli DH5α. Plasmids were reintroduced in U. maydis strain FBD11-21 to confirm the Fuz^+ phenotype. The HindIII–XbaI part was sequenced. Plasmids were stably introduced in U. maydis strain FBD11-21 after linearization with SspI, which removes the ARS sequence. For each plasmid, three independent transformants that displayed a Fuz^+ phenotype were purified and used for pathogenicity tests. The wild-type HindIII–XbaI fragment of plasmids $pE2_{332}$-GAΔH or $pPE2_{332}$-GA was replaced with the HindIII–XbaI fragments of pUM-E2mut plasmids to yield pE2-GAmut# and pPE2-GAmut# plasmids. $pE2_{332}$-GAΔH is a $pE2_{332}$-GA derivative with single XbaI and HindIII sites within the *bE2* ORF. Two additional plasmids, pPE2-GAmutRH and pPE2-GAmutRT, were generated by exchanging SnaBI fragments in plasmids pPE2-GAmut#22 and pPE2-GAmut#34 for the respective SnaBI fragment from pPE2-GAmut#31. All plasmids were introduced by cotransformation with either pGB-$W2_{186}$ or pGB-$W1_{186}$ into yeast strain Y153 and scored for β-galactosidase activity.

For the mutant screen in S. cerevisiae, the mutagenized HindIII–XbaI fragments (see above) were used to replace the respective fragment in $pE2_{332}$-GAΔH to yield pE2-GAmut plasmids. Plasmid pools were introduced into yeast strain Y526, harboring plasmid pGB-$W2_{186}$. Transformants were scored for β-galactosidase expression with the colony assay. To verify the phenotype, plasmid DNA was rescued by transformation in E. coli DH5α and retransformed in S. cerevisiae Y526 (pGB-$W2_{186}$). The HindIII–XbaI part of pE2-GAmut plasmids was sequenced. To make the data comparable to the mutants isolated from the U. maydis screen, HindIII–XbaI fragments were subsequently introduced in pPE2-GA to generate plasmids pPE2-GAmut#. The HindIII–XbaI fragments were also used to replace the respective fragment in pUM-E2 to yield pUME2Ymut# plasmids that were stably integrated into U. maydis FBD11-21, and transformants were scored for their Fuz and Tum phenotypes.

In Vitro Transcription and Translation of *bW*

Transcripts of *bW* were synthesized by incubating 1 μg of EcoRV-linearized plasmid DNA of $pTRbW1_{221}$ or $pTRbW2_{221}$ with 40 U of T7 RNA polymerase (Promega) in a 50 μl reaction as recommended by Promega. About 0.8 μg of the different *bW* transcripts was translated in vitro by using the rabbit reticulocyte lysate system (Promega). Proteins were synthesized in the presence of [^{35}S]methionine in a 25 μl reaction following a standard protocol described by Promega.

Overexpression and Isolation of His-Tagged bE Proteins

Plasmids $pTRbE1_{190}$, $pTRbE2_{190}$, $pETbE2_{190}$T, $pETbE2_{190}$H, and pET15b (control) were transformed into BL21::DE3. Cells were grown in 200 ml of Luria broth containing ampicillin (100 μg/ml) at 37°C to an OD_{600} of 0.1 and then shifted to 28°C. At OD_{600} of 0.5, IPTG was added to 0.2 mM. After 1 hr of incubation, cells were harvested by centrifugation, washed once with buffer A (25 mM Tris–HCl at pH 7.5), and disrupted in buffer B (25 mM Tris–HCl at pH 7.5, 500 mM NaCl, 5 mM imidazole) in a French pressure cell. Inclusion bodies of His-tagged bE proteins were collected by centrifugation (50,000 × g, 4°C, 30 min), washed once with buffer B, and resuspended in buffer B including 6 M urea. After incubation at 4°C overnight with continuous stirring, the extract was centrifuged (see above), and the solubilized bE proteins remaining in the supernatant were stored at −20°C.

These extracts (200 μg of protein) were bound batchwise to 200 μl (bed volume) of Ni^{2+}–Sepharose beads (His-Bind Resin; Novagen) in 1 ml of buffer B including 6 M urea for 1 hr at room temperature under continuous rotation. As a control, Ni^{2+}–Sepharose was incubated with 200 μg of whole-cell extract of BL21::DE3 transformed with pET15b. The resins were poured into small columns and washed with 10 bed volumes each of buffer B, buffer C (25 mM Tris–HCl at pH 7.5, 500 mM NaCl, 50 mM imidazole) and again buffer B, with all buffers containing 6 M urea. Renaturation of the bound proteins was achieved by a sequential washing procedure with decreasing urea concentration from 6 M to 5 M, 4 M, 3 M, 2 M, 1 M, 0.05 M, and no urea in buffer B (5 bed volumes each). The resins were then washed with buffer D (25 mM Tris–HCl at pH 7.5, 200 mM NaCl, 5 mM imidazole) and stored at 4°C in a 1:5 slurry in buffer D. To prove that identical amounts of the different His-bE proteins were bound, 10 μl of the Sepharose beads was boiled in sample buffer (Laemmli, 1970). Proteins were separated on a 12% SDS gel (Lugtenberg et al., 1975) and stained with Coomassie blue.

Protein–Protein Interaction Studies

Ni^{2+}–Sepharose (25 μl) with bound His-bE_{190} protein or bound E. coli whole-cell extract (control) was incubated with 5 μl of in vitro translation mix containing [^{35}S]methionine-labeled $bW1_{221}$ or $bW2_{221}$ protein, respectively, in 300 μl of buffer D for 30 min at room temperature on a rotating wheel. After extensive washing with buffer D followed by buffer C, the Sepharose beads were resuspended in 40 μl of buffer E (25

mM Tris–HCl at pH 7.5, 500 mM NaCl, 750 mM imidazole) and incubated for 30 min at room temperature with continuous rotation. Supernatants containing eluted protein complexes were mixed with sample buffer, boiled, and analyzed by 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography (BioMax MR, Kodak).

Acknowledgments

Correspondence should be addressed to R. K. Details of plasmid construction may be obtained from J. K. at the following e-mail address: uj44211@sunmail.lrz-muenchen.de. We are grateful to S. Fields and P. Bartel for their invaluable help with the two-hybrid system. We thank B. Gelhaus and K.-H. Braun for expert technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft through SFB 190 and through the Leibniz program.

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