The P25 Pathogenicity Factor of *Beet necrotic yellow vein virus* Targets the Sugar Beet 26S Proteasome Involved in the Induction of a Hypersensitive Resistance Response via Interaction with an F-box Protein

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Submitted 2 March 2012. Accepted 5 April 2012.

P25, a *Beet necrotic yellow vein virus* (BNYVV) pathogenicity factor, interacts with a sugar beet protein with high homology to *Arabidopsis thaliana* kelch repeat containing F-box family proteins (FBK) of unknown function in yeast. FBK are members of the Skp1-Cullin-F-box (SCF) complex that mediate protein degradation. Here, we confirm this sugar beet FBK–P25 interaction in vivo and in vitro and provide evidence for in planta interaction and similar subcellular distribution in *Nicotiana tabacum* leaf cells. P25 even interacts with an FBK from *A. thaliana*, a BNYVV nonhost. FBK functional classification was possible by demonstrating the interaction with *A. thaliana* orthologs of Skp1-like (ASK) genes, a member of the SCF E3 ligase. By means of a yeast two-hybrid bridging assay, a direct effect of P25 on SCF-complex formation involving ASK1 protein was demonstrated. FBK transient *Agrobacterium tumefaciens*-mediated expression in *N. benthamiana* leaves induced a hypersensitive response. The full-length F-box protein consists of one F-box domain followed by two kelch repeats, which alone were unable to interact with P25 in yeast and did not lead to cell-death induction. The results support the idea that P25 is involved in virus pathogenicity in sugar beet and suggest suppression of resistance response.

Beet necrotic yellow vein virus (BNYVV) is an economically important pathogen of sugar beet (*Beta vulgaris*) with worldwide distribution. In the presence of this soilborne virus, sugar beet production is only possible by cultivation of resistant genotypes (Johansson 1985). To date, at least two resistance sources, the Holly resistance source *Rz1*, which has been introduced in most cultivars, and *Rz2*, are available for commercial production. Although they are not leading to re-

sistance following virus infection, they can reduce virus replication and mediate a yield and symptom tolerance in the field (Scholten and Lange 2000; Scholten et al. 1999). The characteristic virus-induced symptoms are leaf yellowing, stunting, the formation of a wine-glass-shaped taproot, and an increased number of small lateral roots (rhizomania). Tap root vascular tissue often shows a brown discoloration while, infrequently, yellow leaf veins with necrotic tissue also may occur; both symptoms are indicative of a cell death reaction in the root tissue, which might be a hint for hypersensitive reaction unable to restrict virus spread.

BNYVV consists of four to five single-stranded RNA components. The RNA3-encoded 25-kDa protein (P25) represents the main pathogenicity factor and is responsible for rhizomania symptom formation as well as yield reduction in sugar beet (Koenig et al. 1991; Tamada et al. 1999). The ability to induce necrotic lesions in mechanically inoculated sugar beet leaves and experimental hosts such as *Chenopodium quinoa* is dependent on P25 presence and viral genomic composition (Tamada et al. 1989). Even in *Arabidopsis thaliana* (a nonhost plant), transgenic P25 expression induced a root branching phenotype and hormonal changes (Peltier et al. 2011). This led to the P25 avirulence gene–pathogenicity factor hypothesis, assuming that P25 represents a pathogenicity factor in susceptible and an avirulence determinant in resistant plant genotypes (Chiba et al. 2008, 2011). This hypothesis is supported by the coincidence of the occurrence of sugar beet *Rz1*-overcoming BNYVV isolates in several locations in the United States and Europe (Liu and Lewellen 2007; Liu et al. 2005; Pferdmenges et al. 2009), with P25 proteins harboring specific mutations in the amino acid hypervariable tetrad 67–70 (Bornemann and Varrelmann 2011; Koenig et al. 2009; Schirmer et al. 2005).

The screening of the sugar beet *Rz2* proteasome for P25 interacting partners by means of a yeast two-hybrid (YTH) assay (Thiel and Varrelmann 2009) resulted in the selection of 36 candidates, putatively involved in the virus life cycle or plant resistance, which were assigned to several functional categories. The interaction detected in yeast cells was confirmed in planta for several candidates by means of bimolecular fluorescence complementation (BiFC). Cell death was observed when a cDNA clone with significant homology to the *A. thaliana* kelch repeat containing F-box family protein (FBK) was transiently expressed in *Nicotiana benthamiana* epidermal leaf cells

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Nucleotide sequence data is available in the GenBank database under accession numbers JQ886637.1 for *Beta vulgaris* subsp. *vulgaris* clone F-box/kelch-repeat protein mRNA, JQ886638.1 for *Beta vulgaris* subsp. *vulgaris* clone *Rz1* F-box/kelch-repeat protein mRNA, and JQ886639.1 for *Beta vulgaris* subsp. *vulgaris* clone *Rz2* F-box/kelch-repeat protein mRNA.

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(H. Thiel, *unpublished data*), establishing the need for a more detailed P25 partner characterization. F-box proteins are components of the ubiquitin proteasome system, which is responsible for the selective posttranslational degradation of several cellular proteins (Stone and Callis 2007; Vierstra 2009). Proteins for selective degradation by the 26S proteasome are polyubiquitinated in a three-step process (Hershko and Ciechanover 1986; Vierstra 2009). The final step is catalyzed by E3 ubiquitin ligases belonging to several families, according to their mode of action and subunit composition (Mazzucotelli et al. 2006). The Skp1-Cullin-F-box (SCF) protein complexes, consisting of four major components (S-phase kinase-associated protein 1 [SKP1], Cullin 1 [CUL1], RING-box 1 [RBX1], and an F-box protein) are the most prevalent E3 ligases in plants (Cardozo and Pagano 2004; Ho et al. 2006; Lechner et al. 2006). The F-box proteins determine the selectivity of the SCF complex as they recruit the target proteins by means of a C-terminal protein-protein interaction domain (Cardozo and Pagano 2004). In spite of their key role and sequence diversity (approximately 700 F-box genes have been identified in *A. thaliana*) (Gagne et al. 2002), less than 5% have been functionally characterized (Binder et al. 2007; Chae et al. 2008; Fukamatsu et al. 2005; Han et al. 2004; Kim and Delaney 2002). Because the proteasome seems to be integral to cellular homeostasis by regulation of protein activity through degradation, it is not surprising that F-box proteins possess prominent roles in multiple physiological processes (Yang et al. 2008), including defense (Kim and Delaney 2002; Liu et al. 2002; van den Burg et al. 2008; Xie et al. 1998). Involvement of F-box proteins in the well-characterized defense mechanisms induced by hypersensitive resistance (HR), the systemic acquired resistance (SAR), can be exemplified by *A. thaliana*. SON1, an F-box protein involved in targeting SAR regulators to protein degradation (Kim and Delaney 2002). In tobacco, the F-box protein ACRE189 is necessary for the plant hypersensitive response to *Cladosporium fulvum* Avr9 (van den Burg et al. 2008). Several recent studies have provided evidence for a direct involvement of SCF-complexes in plant-virus interactions, including non-host resistance, virus-induced necrosis, restriction of virus accumulation, and signal transduction in resistance mediated by single dominant genes (Komatsu et al. 2010; Liu et al. 2002; van den Burg et al. 2008). The observation of necrosis induction by the *B. vulgaris* putative FBK protein investigated here suggests that it is either involved in BNYVV resistance or, alternatively, represents a target for P25, required for viral pathogenicity.

In the scope of this study, the full-length F-box coding sequences of BNYVV-susceptible and both *Rz1*- and *Rz2*-resistant sugar beet genotypes were identified and applied for GenBank BLAST analysis, sequence alignment with *A. thaliana* homologues, and comparison of functional domains. YTH interactions and mRNA expression analyses were performed to determine whether there is evidence for an *Rz* resistance-specific P25 interaction that could explain *Rz1* resistance-breaking abilities. Subsequently, *in vitro* and *in vivo* protein interaction assays were carried out to confirm the protein-protein interaction findings in yeast cells. YTH interaction studies with *A. thaliana* SKP1-like (ASK) proteins (Takahashi et al. 2004) were performed to confirm the identity of the sugar beet F-box candidate. Furthermore, a possible effect of the P25-F-box interaction on the E3 ligase complex formation was evaluated. Transient expression in *N. benthamiana* epidermal leaf cells was used to monitor the tissue necrosis and analyze the expression of pathogenesis-related (PR) protein transcripts for HR. In *N. tabacum* leaf tissue, in which tissue necrosis was delayed, P25 and the sugar beet F-box protein were analyzed for subcellular co-localization.

RESULTS

Isolation and sequence analysis of full-length F-box coding sequences from BNYVV-susceptible and BNYVV-resistant sugar beet genotypes.

The *Rz2* cDNA-library-derived F-box-translated amino acid sequence displayed typical conserved domains of FBK proteins (F-box domain and two kelch-repeats) and a stop codon (Thiel and Varrelmann 2009) but its N-terminus, including methionin, was missing. The sequence alignment between a sugar beet expressed sequence tag sequence (from genotype KWS2320, provided by KWS, Einbeck, Germany) and the sugar beet cDNA-F-box sequence identified the missing 5' end of the open reading frame (ORF) (data not shown). The *Rz2*-derived full-length ORF was obtained by reverse-transcription (RT) polymerase chain reaction (PCR) with specific primers and displayed a length of 1,326 nucleotides (nt) which translated into 442 amino acids (aa) (an additional 34 N-terminal amino acids compared with the cDNA encoded F-box candidate), with a calculated molecular mass of 49 kDa. Full-length sequences from susceptible (accession number JQ886637.1) and resistant (*Rz1* [accession number JQ886638.1] and *Rz2* [accession number JQ886639.1]) sugar beet lines were obtained as well.

The full-length sequences of all three alleles displayed the same length and were applied for sequence comparison on the amino acid level (Fig. 1). Polymorphism was detected in 10 positions: three amino acid exchanges between susceptible and *Rz1*, four amino acid exchanges between *Rz1* and *Rz2*, and three amino acid exchanges between *Rz2* and susceptible.

The National Center for Biotechnology Information GenBank database homology BLAST search using BLASTn algorithm (Altschul et al. 1997) was repeated and limited to *A. thaliana* sequences. This resulted in the identification of three FBK proteins displaying significant homology to the *B. vulgaris* FBK candidate (Table 1). Remarkably, Peltier and associates (2011) found three F-box protein messenger RNAs significantly upregulated in transgenic *A. thaliana* expressing BNYVV P25 and displaying a root-branching phenotype (two of them are identical to the sugar beet F-box homologues of this study). The sugar beet FBK consists of one predicted F-box motif and two kelch repeats (Fig. 1) just like the FBK AT1G74510 from *A. thaliana*. Even if conserved residues are clearly visible in the F-box domain depicted in Figure 1, the *A. thaliana* FBK AT2G02870 and AT1G26930 do not lead to prediction of an F-box motif but three kelch repeats (Table 2). Furthermore, thus far, none of the genes identified in the BLAST search showing significant similarity has been functionally characterized. Therefore, indication for a specific function of this *B. vulgaris* FBK is not available yet.

P25 full-length F-box interaction is independent of the sugar beet *Rz* genotype.

Full-length F-box cDNA clones of all three genotypes (*Rz1*, *Rz2*, and susc.) were tested for P25 interaction in yeast cells using LexA-YTH (pEG202-P25 versus pJG4-5-F-box) and compared with the P53-LTA (pEG202-P53 and pJG4-5-LTA) positive control (Iwabuchi et al. 1993; Li and Fields 1993) (Fig. 2A). Yeast expression (Fig. 2E and F) as well as physical P25-F-box interactions were confirmed in all cases (Fig. 2B). The expression level of all full-length F-box proteins appeared lower than those displayed by the shorter cDNA derived from the original library. The strength of the P25-F-box interaction was determined indirectly by quantification of the Leu-driven plasmid-derived green fluorescent protein (GFP) expression relative to a negative control (pEG202-P25 with pJG4-5) and

in comparison with the positive control. No differences in the P25 interaction strength were detectable between the different alleles (Fig. 2D).

BNYVV P25–sugar beet FBK interaction is independent of the P25 amino acid tetrad 67-70 composition.

The results presented above did not give any indication for a plant genotype-specific F-box–P25 interaction; however, the possibility remains that P25 variability might interfere with the interaction. Previous work has shown that P25 protein with va-

line at amino acid position 67 as part of the hypervariable tetrad 67-70 increases BNYVV aggressiveness and mediates resistance breaking (RB) abilities (Koenig et al. 2009), and P25 oligomerization (Klein et al. 2007). To investigate whether P25–F-box interactions are dependent on the P25 hypervariable sequence and probably related to viral aggressiveness, P25-B (₆₇AYHR₇₀) was compared with a P25 of an A-type with RB abilities (from a U.S. isolate; Imperial Valley, CA), P25-RB (₆₇VLHG₇₀) in LexA–YTH (Fig. 2B and C). Similarly to P25-B, P25-RB did not autoactivate the transcription in yeast (Fig. 2Ce)

Fig. 1. Sequence comparison of sugar beet F-box amino acid sequences from different genotypes (*Rz1*, *Rz2*, and susceptible) with National Center for Biotechnology Information BLAST search-selected F-box family proteins from *Arabidopsis thaliana* possessing significant homology. F-box and kelch repeat domains refer to the sugar beet F-box protein and were identified using Simple Modular Architecture Research Tool 5. Arrows represent amino acid exchanges between *Rz1*, *Rz2*, and susceptible F-box sequences.

and was able to interact with the F-box-cDNA candidate at comparable strength (Fig. 2C and D).

The full-length F-box protein is required for P25 interaction.

To obtain a first indication of whether P25 might interfere with F-box substrate recognition or the F-box-SCF complex interaction, LexA-YTH studies were performed with N- and C-terminal parts of the Rz2 full-length F-box protein containing the conserved F-box and kelch domains, respectively. The coding sequences for the N-terminal 187 aa and C-terminal 255 aa, respectively, were reamplified with specific primers and cloned into LexA-BD vector (pJG4-5-ΔN and pJG4-5-ΔC). When tested in YTH assay, both deletion variants did not interact with P25 (Fig. 2B), despite the fact that yeast expression was clearly detectable (Fig. 2E).

Differential expression of the F-box protein in leaves and roots of BNYVV-resistant and BNYVV-susceptible sugar beet genotypes.

To obtain further information about resistance-specific F-box–P25 interaction as well as possible differences in the genotype-specific and virus-induced F-box expression, the mRNA level was analyzed in sugar beet roots and leaves. Susceptible and Rz1 and Rz2 resistant sugar beet genotypes were used for BNYVV natural root infection and mechanical leaf inoculation. At 5 weeks, total RNA samples from local lesions and lateral roots were analyzed by Northern blot hybridization with an F-box antisense probe (Fig. 3). Expression of the F-box gene was only detectable in sugar beet roots in all three genotypes. No indication for genotype-specific or rhizomania (BNYVV and *Polymyxa betae*)-inducible mRNA expression was obtained. Interestingly, we noticed a threefold reduction for the expression of Rz2 F-box mRNA in infected roots.

BNYVV P25 interacts with an FBK from the nonhost *A. thaliana* in LexA-YTH.

A. thaliana F-box cDNA clones which display significant sequence homology to sugar beet FBK AT1G74510 and AT1G26930 (Fig. 1; Table 1) were tested for autoactivation and P25 interaction in LexA-YTH (pEG202-P25–pJG4-5-AT1G74510 and pEG202-P25–pJG4-5-AT1G26930) (Fig. 2B and D). FBK AT1G26930 showed P25 interaction in yeast (Fig. 2B), whereas FBK AT1G74510 did not. Yeast expression (Fig. 2E and F) was clearly detectable (Fig. 2E) in both cases.

The sugar beet cDNA-encoded FBK candidate interacts with BNYVV P25 in vitro.

In order to confirm the F-box–P25 interaction definitely, an in vitro pull-down assay using glutathione-S-transferase (GST)-

tagged F-box was produced in *Escherichia coli*. The in vitro interaction of P25 with Importin-α (Imp-α), as described by Vetter and associates (2004), was used as a positive control. Following the assay, P25 proteins issued from three different sources were similarly immunodetected by the use of either anti-P25, anti-histidine antibodies (data not shown), or Flag antibody (Fig. 4) only on GST–Imp-α and GST–F-box fusion proteins, confirming that F-box protein was interacting as well with P25 in vitro. Moreover, prokaryotic and eukaryotic independent sources of P25 let us favor a direct interaction between the two proteins.

BNYVV P25 interacts with the sugar beet F-box protein in Gal4-YTH.

In order to strengthen the significance of the P25–F-box interaction found in LexA-based YTH, and to perform YTH bridged interaction tests, this assay was additionally performed in a Gal4-based system (Fig. 5). As expected, the oligomerization of P25 (Klein et al. 2007) was retrieved up to 5 mM 3-AT as well as the F-box-P25 interaction (Fig. 5A). F-box-P25 showed the strongest interaction (up to 25 mM 3-AT) when F-box was fused to -AD and P25 to -BD, confirming our previous findings (Fig. 2). The quantification of β-galactosidase (β-gal) activity, estimating the strength of the P25–F-box interaction, resulted in a mean of 16 β-gal Miller units, two times higher than the P53-LTA interaction control (Fig. 5B). The oligomerization of the P25 protein did not give significant activation of the β-gal reporter gene, presumably due to the growth of yeast diploids cells in interaction nonselective conditions.

The sugar beet FBK interacts with the *A. thaliana* SKP1-related proteins ASK1 and ASK2 in yeast.

Although the sugar beet FBK displayed high homology to *A. thaliana* F-box proteins, final evidence for its functional classification was required. Plant F-box proteins interact with SKP1 proteins via their F-box domain (Risseuw et al. 2003; Schumann et al. 2011; Zheng et al. 2002). Out of 19 functional ASK proteins (Takahashi et al. 2004), ASK1 (AT1G10940) and ASK2 (AT5G42190) were selected. Both genes, fused to Gal4-BD in pGBKT7-ASK1 and -ASK2 vectors, respectively (Pazhouhandeh et al. 2006), were used for the Gal-YTH interaction study with the sugar beet FBK (pGAD-F-box) (Fig. 5A). No detectable transcriptional autoactivation was observed for BD-ASK1 and -ASK2 fusion proteins (Fig. 5A). These two components of the SCF complex were not able to interact with P25 (Fig. 5A) but a strong interaction was observed with the sugar beet F-box, up to 15 mM 3-AT (Fig. 5A), slightly lower than the P25–F-box interaction.

Table 1. Comparison of the F-box family protein sequence isolated from an Rz2-resistant sugar beet with sequences from *Arabidopsis thaliana* at the nucleotide level (National Center for Biotechnology Information, GenBank) and on amino acid level (Simple Modular Architecture Research Tool 5)

Accession number	Coverage (%)	Maximal identity (nucleotide) (%)	e Value	Maximal identity (amino acid) (%)	e Value
AT2G02870	78	67	9e ⁻⁸⁹	64	2e ⁻¹⁷²
AT1G26930	74	64	4e ⁻⁴²	59	6e ⁻¹⁶³
AT1G74510	76	65	1e ⁻⁶⁸	55	4e ⁻¹⁷⁶

Table 2. Results of Simple Modular Architecture Research Tool 5 prediction of protein functional domains of sugar beet F-box and its *Arabidopsis thaliana* homologues

Accession number/origin	Functional domain			
	F-box	Kelch repeat 1	Kelch repeat 2	Kelch repeat 3
Bv-F-box-Rz2	97 to 137	191 to 234	235 to 282	...
AT2G02870	...	215 to 259	261 to 307	308 to 356
AT1G26930	...	169 to 212	213 to 260	261 to 316
AT1G74510	94 to 140	193 to 236	237 to 284	...

Fig. 2. LexA–yeast two-hybrid interaction assay of *Beet necrotic yellow vein virus* P25-B-type and resistance breaking (RB) origin with sugar beet and *Arabidopsis thaliana* F-box proteins: Rz2-cDNA and Rz1, Rz2, or susceptible [susc.] full-length clones; Rz2 N- and C-terminal deletion mutants; and *A. thaliana* F-box family proteins (FBK) AT1G74510 and AT1G26930. AD = activation domain, BD = binding domain. **A**, Positive control SV40 LTA (pJG4-5-LTA) with P53 (pEG202-P53). **B**, Specific interaction tests of P25-B (pEG202-P25-B-type) with different F-box proteins (pJG4-5-cDNA, -susc., -Rz1, -Rz2, -ΔN, -ΔC, -AT1G74510, and -AT1G26930); e = empty BD vector test for transcriptional autoactivation. **C**, P25-RB (pEG202-P25-RB) interaction with F-box (pJG4-5-F-box-cDNA). **D**, Relative quantification of Leu-driven green fluorescent protein fluorescence (mean total area density) in diploid yeast expressing the combination of P25 and FBK variants shown in A, B, and C. Standard errors are displayed. **E** and **F**, Immunodetection of LexA and hemagglutinin (HA)-tagged proteins (anti-LexA and -HA) expressed in yeast. Equal polyacrylamide gel electrophoresis loading was checked by staining. Molecular weights are given in kDa. **E**, HA immunodetection in total protein extracts of yeast cells expressing pJG4-5-F-box-cDNA, -F-box-susc., -F-box-Rz1, -F-box-Rz2, -F-box-ΔN, -F-box-ΔC, -AT1G74510, and -AT1G26930. Yeast transformed with empty pJG4-5 served as negative control. **F**, LexA immunodetection in yeast total protein extracts expressing pEG202-P25-B-type, -P25-RB. Yeast with empty vector pEG202 served as negative control.

Interference of P25 with the SCF complex formation.

In the SCF multisubunit structure, CUL1 acts as a scaffold protein that ensures optimal substrate presentation to the E3 ligase, whereas SKP1 (ASK) mediates interaction between the F-box protein and the N-terminal domain of CUL1 (Zheng et al. 2002). To test the hypothesis that P25 interferes with an SCF complex formation involving F-box–ASK–CUL1 subunits assembly, the Gal4-based (-BD and -AD) YTH assay was supplemented with a bridge protein expression vector (pVTU102-U). Different combinations of P25, *A. thaliana* ASK1 and ASK2, sugar beet F-box, and *A. thaliana* CUL1 were tested in the bridging assay to produce binary or ternary complexes; the results are summarized in Table 3.

When ASK1 or ASK2 were fused to the BD, the known interaction with CUL1 was clearly demonstrated (Table 3, numbers 3 and 4) and was enhanced in the presence of the F-box protein (Table 3, numbers 11 and 12), suggesting a conformational change mediated by the F-box interaction. When CUL1 was fused to BD, only the ASK2-CUL1 interaction was detected (Table 3, number 6), ASK1-CUL1 was not detected (Table 3, number 5), even in the presence of the F-Box protein (Table 3, number 9) that enhanced the ASK2-CUL1 interaction (Table 3, number 10). However, efficient ternary complexes between CUL1, ASK1 and ASK2, and the F-box proteins were retrieved when CUL1 was fused with the AD domain (Table 3, numbers 7 and 8). The binary complexes F-box–ASK1 or F-box–ASK2 (Table 4, numbers 18, 20, 22, and 24) were not affected when the P25 protein was expressed via the bridge vector (Table 4, numbers 19, 21, 23, and 25). Similarly, binary complexes between F-box and P25 were not affected by the expression of ASK1 or ASK2 protein via the bridge vector

(Table 4, numbers 34 to 37), suggesting the absence of overlapping interaction domains. However, when ternary complex formation between ASK–F-box–P25 proteins were assayed in YTH (Table 4, numbers 26 to 31), a complex was detectable only when ASK2 was used (Table 4, numbers 29 and 33) but was never found with ASK1 (Table 4, numbers 27 and 31). As controls, several autoactivation tests were performed (Table 3, numbers 1 and 2 and Table 4, numbers 13 to 17) to validate our results.

The sugar beet F-box protein induces an HR reaction in *N. benthamiana* leaves.

The P25 ORF and F-box cDNA were cloned into binary vectors for agrobacterium mediated BiFC assay and applied for transient expression in *N. benthamiana* leaf tissue to confirm the interaction in planta. Remarkably, the F-box candidate induced necrosis already at 24 h postinfiltration (hpi). Due to the speed by which the symptoms occurred, the necrosis let us suspect an HR reaction. To confirm that the F-box protein alone and not the translational fluorescent marker-F-box fusion was responsible for the observed effect, the F-box-cDNA candidate and all three F-box full-length sequences were cloned into a binary plant expression vector (pBin). Subsequent agroinfiltration in *N. benthamiana* exactly reproduced necrotic cell death in the infiltrated tissue, starting at 24 hpi. When the construct was used for transient expression in sugar beet leaves, leaf yellowing was observed starting at 18 days postinfiltration (dpi). *N. benthamiana* leaves (3 dpi) and sugar beet leaves (21 dpi) following treatment with the empty vector pBin and pBin–F-box–Rz2, respectively, are shown in Figure 6. In order to verify whether P25 interferes with the induction of necrosis, P25 was used for co-infiltration with F-box–Rz2, resulting in no visible difference in time of occurrence and severity of the necrotic reaction compared infiltration with F-box–Rz2 alone (data not shown). The F-box agroinfiltration experiment was repeated with leaves of resistant and susceptible sugar beet genotypes to clarify whether the yellowing tissue occurrence was Rz specific; however, no phenotypic differences were ob-

Fig. 3. Northern blot analysis for tissue-specific detection of F-box-mRNA expression in different *Beta vulgaris* breeding lines depending on infection with *Beet necrotic yellow vein virus*. **A**, Rub inoculated leaves (14 days postinoculation) and **B**, roots from 6-week-old sugar beet plants growing in B-type soil (Germany). Numbers below blots display total area densities relative to loading controls. Comparative loading of the gel was verified by ethidium bromide staining of 18S RNA.

Fig. 4. In vitro pull-down assays to test for P25 in vitro interaction with F-box. Flag-P25 purified from *Baculovirus* sp.-infected Sf9 cells was incubated with glutathione-S-transferase (GST) alone, GST-importin α (rice), and GST-F-box. GST pull-down of P25 was visualized by the detection of the viral protein using flag antibody after immunoblotting. Reactions performed with in vitro synthesized histidine-tagged P25 gave identical results (data not shown).

Fig. 5. A, Gal4-based yeast two-hybrid (YTH) interaction assay of *Beet necrotic yellow vein virus* P25 (pGAD424-P25 and pGBT9-P25), sugar beet F-box candidate (pGAD424- and pGBT9-F-box-cDNA), and *Arabidopsis thaliana* SKP1-like (ASK)1 and ASK2 (pGBKT7-ASK1 and pGBKT7-ASK2). Both vectors pGBT9 and pGBKT7 contain the coding region of the DNA-binding domain (BD) while pGAD424 carries the Gal4 activation domain (AD). YTH interaction assay of candidates with empty vectors pGAD424 (e), pGBT9 (e1), and pGBKT7 (e2) to test for autoactivation. YTH interaction assays were plated on selective media lacking different combinations of amino acids tryptophan (W), leucine (L), and histidine (H). The positive control with P53 (pVA3) and LTA (pTD1) was performed on WL and WLH media. YTH was carried out in the presence of 1, 5, 15, and 25 mM 3-aminotriazole (AT) to suppress background growth on WLH medium. **B**, Quantification of β -galactosidase (β -gal) activity in Miller units of yeast diploids expressing the interaction partners grown nonselective conditions in liquid culture assay at 30°C. Assays were performed in triplicate sets, with three independent cultures for each strain. Standard errors are displayed.

served (data not shown). Although agroinfiltration with pBin did not induce the tissue necrosis, the possibility remained that the agroinfection process might have interfered with it. Transient expression via particle biolistic with co-expression of a fluorescent marker was chosen to exclude this possible *Agrobacterium tumefaciens* effect. *N. benthamiana* leaves were used for co-bombardment with pBin-F-box-Rz2 plus the gene for DsRed under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (pe35-DsRed) or pBin-F-box-Rz2 alone. The combination of pe35-DsRed plus pBin was co-bombarded as control. At 3 dpi, five leaves of each treatment were analyzed under epifluorescence microscopy. Bombardment with F-box cDNA alone resulted in the detection of 67 single epidermal cells showing autofluorescence induced by necrosis indicative for cell death, whereas no cells displaying DsRed fluorescence were detected. Co-bombardment of the F-box plus DsRed resulted in 87 autofluorescent and 7 red-fluorescing cells. By contrast, bombardment with DsRed alone led to 131 cells showing red and only 5 cells displaying autofluorescence, demonstrating that i) a high percentage of cells was hit with both plasmids and ii) transient DsRed expression was suppressed when F-box protein was co-expressed. These observations suggested that, with or without agrobacteria, the expression of sugar beet F-box protein induced cell death in *N. benthamiana* leaf cells.

Transient F-box protein expression induces the accumulation of PR protein transcripts in *N. benthamiana* leaf tissue.

To find evidence that the rapid necrosis induced by transient F-box expression in *N. benthamiana* leaf tissue was associated with an HR reaction, the accumulation of PR protein transcripts was assayed. *N. benthamiana* leaf tissue was agroinfiltrated with pBin-F-box-Rz2 and the empty pBin vector as control. Samples were taken 24 and 36 hpi for extraction of total RNA. Specific primers for the *N. tabacum* PR gene *PR-1c* were used in semiquantitative RT-PCR reactions (Fig. 7), using the constitutively expressed elongation factor 1 α (*EF1 α*) housekeeping gene as an internal control.

Densitometric analysis of the RT-PCR signals highlighted an increased mRNA expression level of *PR-1c*, relative to tissue infiltrated with pBin that was detected already at 24 hpi

Table 3. Analyses of protein binary and ternary complex formation in yeast two-hybrid supplemented with a bridge protein expression vector to evidence for F-box interaction within members of the Skp1-Cullin-F-box complex (*Arabidopsis thaliana* SKP1-like [ASK] 1 and 2 and Cullin 1 [Cul1])

Number	BD ^a	Bridge ^b	AD ^c	Interaction ^d
1	F-box	Ø	CUL1	–
2	CUL1	Ø	F-box	–
3	ASK1	Ø	CUL1	+
4	ASK2	Ø	CUL1	+
5	CUL1	Ø	ASK1	–
6	CUL1	Ø	ASK2	+
7	F-box	ASK1	CUL1	+
8	F-box	ASK2	CUL1	+
9	CUL1	F-box	ASK1	–
10	CUL1	F-box	ASK2	++
11	ASK1	F-box	CUL1	++
12	ASK2	F-box	CUL1	++

^a Protein fused to Gal4 DNA-binding domain.

^b Bridge protein expressed from pVTU102-U vector; Ø = empty vector control.

^c Protein fused to Gal4 activation domain.

^d Symbols: + = weak growth, below 1 Miller units; ++ = average growth, 1 to 2 Miller units; +++, over 2 Miller units; – = absence of growth.

(accumulation ratios were 1.5 for pBin and for 7.1 pBin-F-box-Rz2). Such differential expression increased at 36 hpi (accumulation ratio of 5.7 for pBin and 13.6 for pBin-F-box-Rz2), demonstrating that the beet F-box protein expression in leaves is associated with the induction of HR in *N. benthamiana*. The experiment was performed in different combinations: F-box-Rz2–P25, P25 alone, and F-box-Rz2 alone. The necrotic cell death was induced with and without P25. P25 did not have any influence on the reaction. As for YTH interaction studies, we tested whether the HR induced by the full-length F-box protein could be ascribed to one of the two functional domains of the protein. F-box- Δ N and F-box- Δ C parts were transiently agro-expressed in *N. benthamiana* leaf tissue. No occurrence of necrotic cell death was observed with both fragments, suggesting the exclusive HR induction by a functional F-box protein.

Subcellular co-localization of P25 and F-box.

Different *A. thaliana* F-box proteins localize to either the cytoplasm or the nucleus, which is believed to define the incorporation into specific SCF complexes (Schumann et al. 2011). *Agrobacterium tumefaciens*-mediated co-expression of F-box and P25 fused to fluorescent marker proteins in *N. benthamiana* epidermal cells should allow us to determine sugar beet F-box subcellular localization and indicate whether the physical interaction might affect the F-box function. Subcellular co-localization was examined with translational fusions of P25 and the sugar beet F-box protein to GFP (P25-GFP) and monomeric red fluorescent protein (mRFP-F-box), respectively, by means of transient agroexpression in *N. tabacum* epidermal cells (Fig. 8). Expectedly, when assayed alone, P25 localized in the cytoplasm and the nucleus, as described by Vetter and associates (2004) (Fig. 8C). A similar subcellular distribution was observed for the F-box protein (Fig. 8D). The co-expression

Table 4. Analyses of protein binary and ternary complex formation in yeast two-hybrid supplemented with a bridge protein expression vector to evidence for P25 interaction within members of the Skp1-Cullin-F-box complex (*Arabidopsis thaliana* SKP1-like [ASK] 1 and 2 and F-box).

Number	BD ^a	Bridge ^b	AD ^c	Interaction ^d
13	Ø	Ø	Ø	–
14	F-box	Ø	Ø	–
15	P25	Ø	Ø	–
16	P25	F-box	Ø	–
17	Ø	F-box	ASK2	–
18	ASK1	Ø	F-box	+++
19	ASK1	P25	F-box	+++
20	ASK2	Ø	F-box	+++
21	ASK2	P25	F-box	+++
22	F-box	Ø	ASK1	+++
23	F-box	P25	ASK1	+++
24	F-box	Ø	ASK2	+++
25	F-box	P25	ASK2	+++
26	ASK1	Ø	P25	–
27	ASK1	F-box	P25	–
28	ASK2	Ø	P25	–
29	ASK2	F-box	P25	++
30	P25	Ø	ASK1	–
31	P25	F-box	ASK1	–
32	P25	Ø	ASK2	–
33	P25	F-box	ASK2	+++
34	P25	ASK1	F-box	+++
35	P25	ASK2	F-box	+++
36	F-box	ASK1	P25	+++
37	F-box	ASK2	P25	+++

^a Protein fused to Gal4 DNA-binding domain.

^b Bridge protein expressed from pVTU102-U vector; Ø = empty vector control.

^c Protein fused to Gal4 activation domain.

^d Symbols: + = weak growth, below 1 Miller units; ++ = average growth, 1 to 2 Miller units; +++, over 2 Miller units; – = absence of growth.

and overlay of the red and green fluorescence channel gave evidence for co-localization (Fig. 8E to J) and did not visibly interfere with the subcellular distribution of both proteins after 3 dpi. The fusion proteins of the expected size were detected in Western blots of total protein extracts by means of either anti-hemagglutinin (HA) or anti-c-myc antibodies (data not shown).

DISCUSSION

In the work presented here, we have proven the identity of a sugar beet F-box protein containing two C-terminal kelch repeats, which physically interacts with the BNYVV main pathogenicity factor P25. The interaction was reproducibly detected in yeast cells (with LexA- and Gal4-based transcription activation), in vitro, and in planta. Two additional observations have been made here, providing evidence for an interaction in planta and supporting the final conclusion that P25 and F-box, indeed, “meet” in sugar beet root cells. The F-box protein is constitutively expressed in roots, the main tissue where BNYVV replicates, and both proteins show a similar subcellular distribution.

Although the diversity of kelch repeat containing F-box proteins in *A. thaliana* is high, only four of them have been functionally characterized (Schumann et al. 2011). They all possess key functions in light signaling, photomorphogenesis, and regulation of the circadian clock (Demarsy and Fankhauser 2009; Harmon and Kay 2003; Kim et al. 2007; Sawa et al. 2007). All *A. thaliana* FBK homologue to the P25-interacting

sugar beet FBK identified here have not been functionally characterized yet, suggesting that their specific roles and their ubiquitination substrates are unknown as well. The interaction of P25 with FBK AT1G26930 but not with AT1G74510 from a nonhost supports the observations of P25 interference with the ubiquitin proteasome system. If the *A. thaliana* FBK–P25 interaction interferes with the nonhost properties, it needs to be analyzed with knock-out mutants in further work. The main question to be discussed is: what are the consequences of this interaction for the host or the pathogen? Evaluating all the results obtained in this study, there is no indication of a direct effect of this interaction on BNYVV resistance in sugar beet determined by the presence of the major dominant resistance genes *Rz1* or *Rz2*. The interaction is not allele specific and no clear resistance-specific F-box mRNA expression was detected, except for *Rz2*-specific reduction in FBK mRNA in infected roots. Furthermore, P25 mutations, which determine *Rz1*-RB abilities, did not interfere with the F-box interaction either.

Several indications were obtained for involvement of this sugar beet FBK in plant defense and induction of HR, as shown by rapid necrosis induction in *N. benthamiana* cells following agroexpression, including increased *PR-1c* mRNA expression and sugar beet leaf yellowing. The transient DsRed expression assay following F-box co-bombardment supported the rapid cell-death induction in *N. benthamiana* leaf cells, thereby excluding a possible agrobacteria effect. It remains elusive whether P25 has the ability to inhibit a possible resistance induction mediated by the FBK in root cells. However,

Fig. 6. Phenotype of *Nicotiana benthamiana* (*N.b.*) and *Beta vulgaris* (*B.v.*) leaf tissue response at 3 and 21 days postinfiltration (dpi), respectively, following transient *Agrobacterium*-mediated expression of *Rz2* sugar beet F-box protein (binary plant expression vector pBin-F-box-*Rz2*). Agroinfiltration of an empty vector served as control (pBin).

Fig. 7. Analysis of pathogenesis-related (PR) protein gene (*PR-1c*) expression in *Nicotiana benthamiana* leaf tissue agroinfiltrated with F-box-*Rz2* (binary plant expression vector pBin-F-box-*Rz2*) and control infiltrated with pBin vector means of semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (18, 23, 28, and 33 cycles) at 24 and 36 h postinfiltration (hpi). RT-PCR amplification of constitutively expressed elongation factor 1α (*EF1α*) transcripts was applied as a quantitative standard. Results were independently reproduced two times. Numbers below the gels display total area densities of the different RT-PCR signals that served to establish the accumulation ratio presented in the text.

Fig. 8. Subcellular localization following transient agroexpression of *Beet necrotic yellow vein virus* P25 and *Beta vulgaris* F-box fused to green fluorescent protein (GFP) and monomeric red fluorescent protein (mRFP) protein, respectively, in leaf epidermal cells of *Nicotiana tabacum* at 3 days postinoculation. **A**, Binary plant expression vector pBin-empty-GFP. **B**, pBin-mRFP-empty (control). **C**, pBin-P25-GFP. **D**, pBin-mRFP-F-box. **E-J**, Co-localization pBin-mRFP-F-box and pBin-P25-GFP. **A**, **C**, **E**, and **H** represent the green detection channel; **B**, **D**, **F**, and **I** represent the red channel; and **G** and **J** show the overlay of both channels.

rapid necrosis, which was induced by the transient high-level 35S-driven FBK agroexpression in *N. benthamiana* leaves, was not inhibited or delayed by P25 co-expression. Nevertheless, necrosis and yellowing are two phenotypes that are associated with BNYVV leaf symptoms and seldom occur in field infections and in greenhouse infection assays. In contrast, FBK expression was not detectable in local lesions in sugar beet leaves. This suggests a root-specific effect of the P25 protein but the detailed mode of action remains unidentified. HR and transient expression assays for sugar beet roots have to be developed to analyze this in more detail.

Several viruses developed mechanisms to recruit host target proteins for degradation to the 26S proteasome. The silencing suppressor protein P0 of the poleroviruses contains an F-box motif at the N terminus (Pazhouhandeh et al. 2006) and the authors demonstrated that P0 targets a component of the RNA silencing to the SCF complex. In contrast, the movement protein of *Tobacco mosaic virus* is degraded following ubiquitination, and Reichel and Beachy (2000) suggested that the virus uses the process to control virus movement via limiting the damaging influence of the movement protein on the structure of the cortical endoplasmic reticulum. Several plant F-box proteins and other E3 ubiquitin ligase components have been described being involved in plant defense, including response to virus infections (Dreher and Callis 2007; Komatsu et al. 2010; van den Burg et al. 2008); however, none of them represents an FBK or shows significant sequence homology to the sugar beet FBK characterized in this study. Like all other viruses, BNYVV relies on the host cell for replication and for creation of a suitable environment to elude defense mechanisms and anticipate the cellular machinery. This process involves protein–protein interactions, most likely including those with the ubiquitin pathway. It has already been shown that (plant) viruses encode their own ubiquitination components (Aronson et al. 2000; Baumberger et al. 2007; Bortolamiol et al. 2007) or affect SCF via the COP9 signalosome (Lozano-Duran et al. 2011; Randow and Lehner 2009). Hence, the main question in the scope of this study was if and how BNYVV-encoded P25 modifies the plant ubiquitination machinery or if, alternatively, the host might specifi-

cally use the ubiquitin–proteasome system to counteract the virus infection. No hint was obtained for P25 being a substrate of this FBK. First, the only P25 lysine is not embedded in a consensus ubiquitylation motif. Second, no ladderized signals were obtained in Western blot experiments. Finally, P25 has never been found after immunoprecipitation and during proteomic studies (D. Gilmer, *unpublished*). A clear and specific P25 binding to the substrate-binding domain (kelch domain) as well as resistance-specific interaction were not observed. Taken together, we anticipate that BNYVV replication is not affected by this interaction. In contrast, evidence was found for a direct effect of P25 on an SCF complex formation involving ASK1 protein. Indeed, the YTH assay, supplemented with an additional expression vector, allowed bridging experiments. At first, the results did support the F-box identity as F-box–ASK1–CUL1 complexes and demonstrated the functionality of the system even if the enzymatic assays displayed lower Miller unit values due to lower protein expression linked to the presence of three plasmids and the nature of the yeast strains used. However, on the one hand, the F-box–P25 interaction was not affected by co-expression of ASK1 or ASK2 whereas, on the other hand, no abolishment of the F-box–ASK1 or SK2 binding by P25 was observed, indicating that proteins bind together with distinct domains. When a complex formation of all three components was required for transcriptional activation, this did occur in the case of only ASK2 but not ASK1, an indication of a competition or interference of physical binding. One possibility is that the F-box–P25 complex is no longer able to bind ASK1 but interacts with ASK2, or that the ASK1–F-box complex cannot bind P25. Assuming an effect of the viral pathogenicity factor, P25 seems to inhibit the ASK1–F-box binding or inhibit the ASK2–F-box complex function as a competitor to the natural substrate. Whether this happens by modification of the structure or via another mechanism remains speculative. However, this might result in the prevention of F-box substrate presentation to the SCF complex and suggests that P25 interferes with the degradation of the sugar beet F-box substrate (Fig. 9).

The ways by which plant virus proteins interfere with the 26S proteasome are quite diverse and, although a few studies

Fig. 9. Model depicting the possible effects of P25-F-box interaction on the Skp1-Cullin-F-box complex formation and target degradation. **A**, Sugar beet F-box target binds to the substrate domain or kelch-repeats (K) of the F-box protein, followed by E2-mediated attachment of ubiquitin molecules (U) and subsequent 26S proteasome (26S) degradation. When expressed ectopically in leaves, hypersensitive reaction (HR) is induced. The cellular function of the root-specific constitutive expression is unknown. **B**, Interference of P25 with F-box *Arabidopsis thaliana* SKP1-like complex formation might lead to either increased or reduced target activity (ubiquitination and degradation). Possibly, rhizomania symptom formation is affected by this process.

exist which show at least some similarities, the final understanding of how P25 exerts its function as a pathogenicity factor is still missing. Further experiments will aim to verify whether ASK2–FBK–P25 could represent a stable and metastable F-box–ASK interaction that could, on the one hand, abrogate the F-box function and, on the other hand, deplete the cell of the ASK proteins recruited in such interaction. Investigations are also needed to ascertain whether stable overexpression or RNAi-based knock-outs in transgenic sugar beets using full-length and deletion constructs are useful in determining if interference with resistance against BNYVV or the response to infection with other pathogens can be generated. Summarized, this analysis provides a good basis to clarify the unknown BNYVV resistance mechanism.

MATERIALS AND METHODS

Construction of F-box and P25 yeast expression vectors.

All cloning procedures and manipulations were performed following standard techniques according to Sambrook and Russell (2001) unless specified otherwise. The yeast vector pJG4-5 (MoBiTec GmbH, Göttingen, Germany) carrying the partial F-box-cDNA encoding sequence (pJG4-5-cDNA) was obtained from a P25-B YTH screen of a sugar beet (*Rz2*) cDNA library as described previously (Thiel and Varrelmann 2009). Additionally, a coding sequence of a P25 variant from an *Rz1* RB isolate (Imperial Valley, Salinas, CA, U.S.A.) (supplied by Syngenta Seeds AB, Landskrona, Sweden) (Liu et al. 2002) was PCR amplified and cloned into pEG202 (MoBiTec GmbH) as described (Thiel and Varrelmann 2009). Similarly to P25-B (₆₇AYHR₇₀), P25-RB (₆₇VLHG₇₀) did not autoactivate transcription in yeast (Fig. 2C) and interacted with a cDNA-encoded F-box at comparable strength.

RT-PCR from total RNA of healthy sugar beet breeding lines (MS150, susceptible and MS298, *Rz1* resistant, supplied by Syngenta Seeds AB; and 8T_0015, *Rz2* resistant, supplied by KWS) with specific oligonucleotides (5′-ATGAATCTTGGAAGATCTTTCATTTC-3′ and 3′-ATCTCGAGTTAGCAGCCCATTATTGCAC-5′; restriction sites introduced are underlined) led to amplification products that were further introduced into pJG4-5. Resulting plasmids were named pJG4-5-F-box-susc., pJG4-5-F-box-*Rz1*, and pJG4-5-F-box-*Rz2*, respectively. To confirm the cDNA library, F-box P25 interaction in Gal4 YTH, the F-box-cDNA, and the P25 coding sequence were subcloned into the plasmid pGBKT7 and pGAD424 (Clontech, Palo Alto, CA, U.S.A.) using *EcoRI* and *Sall*. The pGBT and pGAD-P25 vectors have been described previously (Klein et al. 2007). pVT-U102 (Vernet et al. 1987) were cloned using the GATEWAY system (Invitrogen, Darmstadt, Germany).

Construction of *Rz2*-derived F-box N- and C-terminal deletion mutants, encoding the 187 N-terminal F-box domain-spanning amino acids and C-terminal 255 aa, including the two kelch motifs, was carried out as follows: both 5′ 561-nt and 3′ 765-nt fragments were amplified with the corresponding full-length primers (discussed above) together with specific oligonucleotides (5′-low: 3′-TATCTCGAGCTAAACCGCAAGTGACTCCTTATCC-5′ and 3′-up: 5′-ATGAATTCGGTACCGAAGCTTTGGTCTTTC-3′). Both PCR fragments were cloned into pJG4-5, resulting in pJG4-5-F-box-ΔN and pJG4-5-F-box-ΔC, respectively.

A. thaliana FBK cDNA full-length ORF accession numbers AT1G74510 and AT1G26930 (provided by M. Quint, Leibniz Institute of Plant Biochemistry, Halle, Germany) were amplified with specific oligonucleotides using *EcoRI* and *XhoI* and cloned in pJG4-5. Resulting plasmids were named pJG4-5-AT1G74510 and pJG4-5-AT1G26930.

Construction of binary vectors

for transient expression and subcellular localization studies.

For transient expression in *N. benthamiana* leaf tissue, all F-box full-length and F-box deletion sequences described above were PCR amplified to introduce appropriate restriction sites and were cloned under control of the CaMV 35S promoter (35S) and pA signal into a binary vector pBin19 (Bevan 1984) (modified for direct subcloning with *EcoRI* and *XhoI*), resulting in pBin-F-box-cDNA, pBin-F-box-susc., pBin-F-box-*Rz1*, pBin-F-box-*Rz2*, pBin-F-box-ΔN, and pBin-F-box-ΔC.

For subcellular localization studies, the sugar beet F-box and BNYVV P25 were fused to mRFP1-Q66T and GFP as described previously (Haseloff et al. 1997; Jach et al. 2006) to obtain N- and C-terminal translational fusions, respectively. Additionally, a c-myc tag and an HA tag plus a linker sequence (consisting of four glycine and one serine residues) as described by Schmiedl and associates (2000) were inserted between the fusion partners. Marker genes were PCR reamplified, thereby introducing tag and linker sequences, and cloned into binary vectors under the control of an enhanced CaMV 35S promoter and a polyA terminator cassette. This resulted in pBIN19-mRFP-c-myc and pBIN19-HA-GFP. The BNYVV P25 and sugar beet F-box coding sequences were PCR amplified with specific primers and cloned into pBIN19-HA-GFP and pBIN19-mRFP-c-myc, respectively. Plasmid inserts were verified by sequencing. Confocal imaging of GFP- and mRFP-expressing leaf tissues was performed using a Leica TCS SP5 confocal imaging system with excitation and emission wavelengths of 488 and 510 to 515 nm for GFP and 514 to 561 and 600 to 630 nm for both DsRed and mRFP, respectively.

Protein–protein interaction in vitro GST pull-down assay.

Histidine-tagged BNYVV B-type P25 was cloned into plasmid pVEX 2.3 (In Vitro Expression; Roche Molecular Biochemicals, Meylan, France) and was expressed by a rapid translation system (RTS100; Roche Molecular Biochemicals). Flag-P25 was produced in *Sf9* insect cells infected with a recombinant *Baculovirus* sp. F-box candidate cDNA PCR was amplified with specific primers 5′-AAAGGATCCATGGAAGTCGATTTTC-3′ and 5′-AAAGAAATTCAGTCCAAGTCCGTCC-3′ and the amplicon cloned into pGEX 2TK (GE Healthcare, Munich) vector to obtain pGEX-F-box using *BamHI* and *EcoRI*. The in vitro GST pull-down assays was performed according to Vetter and associates (2004). Finally, aliquots of the bound protein were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were immunodetected using specific P25, histidine, or flag antibodies.

Yeast interaction assays of sugar beet F-box candidate with *A. thaliana* ASK1, ASK2, and Cull1.

A. thaliana pGBTk7-ASK2 (accession number AT5G42190), pGBTk7-ASK1 (accession number AT1G10940), and Cullin1 (CUL1, accession number AT4G02570) clones were from P. Genschik's lab (Institut de Biologie Moléculaire des Plantes [IBMP], Strasbourg, France).

Sequence analysis.

All inserts in clones were sequence verified using standard primers. Sequencing reactions were carried out by Eurofins MWG Operon (Ebersberg, Germany) or by M. Alioua (IBMP), using a BigDye terminator sequencing kit (Applied Biosystems, Villebon sur Yvette, France) and specific primers with a Hitachi 3100 Genetic Analyzer (Applied Biosystems).

Sequence alignments with ClustalW algorithms (Thompson et al. 1994) and assembly of full-length F-box cDNA sequences were carried out using DNASTAR lasergene software

(GATC Biotech AG, Konstanz, Germany). In silico manipulations of plasmids and primer design were performed with VectorNTI software (Invitrogen). Identification of F-box functional domains was carried out using the "Simple Modular Architecture Research Tool" (Letunic et al. 2004; Schultz et al. 1998).

Yeast strains, media, and YTH systems.

Two different YTH systems were used in this study. The Grow 'n' Glow YTH system (MoBiTec GmbH) is a modified version of the LexA/B42 YTH system (Fields and Song 1989). It consists of pEG202 as bait and pJG4-5 as prey vector (Gyuris et al. 1993) and an additional LexA operator-controlled GFP reporter plasmid pNGI1 (Cormack et al. 1998). All yeast transformations, media preparations, and mating procedures were carried out as described by Thiel and Varrelmann (2009). Total area optical density analysis of GFP fluorescence was carried out by epifluorescence microscopy of yeast cells with Gel-Pro analyzer software 6.0 (Media Cybernetics, Bethesda, MD, U.S.A.) (Thiel and Varrelmann 2009).

The Gal4-based YTH system (Clontech) consists of pGAD424 (Clontech) as bait and pGBT9/pGBKT7 as prey plasmids. The YTH procedures were performed by using the Matchmaker two-hybrid system based on the Yeast Protocol Handbook (BD Biosciences; Clontech) and as described previously (Klein et al. 2007; Link et al. 2005). The HF7c strain was transformed with Gal4-BD-p25 fusions (pGBT9; Clontech) and Gal4-BD-ASK1 and Gal4-BD-ASK2 (pGBKT7; Clontech), whereas the Y187 strain was transformed with bait plasmids (Gal4-AD-P25 or -F-box fusions). Mating was used to obtain diploids expressing both fusion proteins. Interactions were identified by the ability of diploids to grow on minimal medium depleted with tryptophan, leucine, and histidine and in the presence of X- α gal or 3-aminotriazole (AT) at 1 to 25 mM. The strength of the yeast interaction was quantified using a β -gal liquid culture assay with o-nitrophenyl β -D-galactopyranoside as substrate (optical density at 420 nm), displayed as β -gal units (Yeast Protocols Handbook; Clontech protocol).

In bridging assays, ASK1, ASK2, F-box, or P25 were expressed from pVT-U102 and carried out in AH109 strain. Yeast growth was selected on dropout plates lacking adenine, histidine, tryptophan, leucine, and uracil (–AHWLU).

Immunodetection of LexA and HA-tagged proteins in yeast.

Protein extraction from yeast cells and the immunodetection procedure were carried out as described previously (Thiel and Varrelmann 2009), with some modifications. The protein samples were separated by 12% SDS-PAGE and electroblotted on nitrocellulose (GE Healthcare Europe GmbH, Freiburg, Germany) for LexA detection or polyvinylidene difluoride (Roche Molecular Biochemicals) membranes for HA detection.

Plant material and plant growth conditions.

N. benthamiana and *N. tabacum* plants were cultivated in a glasshouse with day length of 14 h under continuous artificial light at 22°C in a mixture of peat and sand (1:1). Plants of BNYVV-susceptible and BNYVV-resistant (*Rz1* and *Rz*) sugar beet lines (supplied by KWS) were grown in a mixture of peat and sand (1:1) and a German field soil sample infected with rhizomania pathotype B for 5 weeks in the greenhouse at 24°C (day) and 18°C (night) with a day length of 14 h under continuous artificial light. Sugar beet leaves were mechanically rub-inoculated with sap obtained from *Chenopodium quinoa* local lesions induced by BNYVV.

Transient expression following agroinfiltration and particle bombardment of F-box candidates.

Binary vectors were mobilized into *Agrobacterium tumefaciens* C58C1 (pGV2260) and cultures were prepared for agroinfiltration as described (Voinnet et al. 2003), with an optical density at 600 nm adjusted to 1.0. The DsRed coding sequence (Clontech) under control of 35S and pA (pe35-DsRed, original pDsRed-C1; Clontech) was applied for microprojectile co-bombardment with pBin-F-box-*Rz2* and pBin on detached leaves of 4- to 5-week-old *N. benthamiana* plants using a particle inflow gun (Gray et al. 1994). At 3 dpi, five leaves of each treatment were rated for single epidermal cells displaying DsRed expression using epifluorescence microscopy (Leica DMR) with a DsRed filter (Emitter HQ 620/60, Beamsplitter Q 585 LP, and Exciter HQ 565/30). Dead cells were visualized by the displayed autofluorescence using a GFP filter (Emitter D 510/40, Beamsplitter 425 DCLP, and Exciter D 395/40). Photographs were taken using a Leica DFC camera, DFC300 FX.

Detection of PR protein transcripts.

Total RNA from *N. benthamiana* agroinfiltrated leaf tissue was prepared as described previously (Puthoff and Smigocki 2007) and treated with DNase (RQ1; Promega GmbH, Mannheim, Germany). Primers specific for *N. tabacum PR-1c* (X05454) (Pfitzner and Goodman 1987) and the constitutively expressed translation *EF* gene (*EF1 α* , AF120093) as internal control (Zhang et al. 2004) were applied for semiquantitative RT-PCR. cDNA was generated using AMV reverse transcriptase (Promega GmbH) and an oligo-dT primer. Calculated product sizes were 358 and 495 bp for *PR-1c* and *EF1 α* , respectively. The PCR conditions were 75°C for 5 min, 94°C for 30 s, annealing at 52°C for 30 s, and extension at 68°C for 30 s. PCR products were checked on a 1.5% agarose gel after 18, 23, 28, and 33 cycles. Total area optical density analysis RT-PCR products relative to the background was carried out using ImageJ software 1.45i (National Institute of Health, Bethesda, MD, U.S.A.). Analysis was performed two times from two independent RNA extractions and resulted in PCR products of comparable densities.

F-box mRNA expression analysis.

For tissue-specific and rhizomania-induced F-box mRNA expression, total RNA extracts (Hamilton et al. 2002) were derived from roots of 5-week-old sugar beet plants grown in natural field soil infected with BNYVV B-type. Leaf RNA was extracted from BNYVV local lesions (14 dpi) from sugar beet leaves. A 1,040-bp fragment of the F-box gene was cloned in antisense orientation under the control of a T7 promoter for generation of an in vitro transcript (RiboMAX Large-Scale RNA Production System; Promega GmbH). Northern blot analyses of F-box mRNA were performed as described previously (Varrelmann et al. 2007).

ACKNOWLEDGMENTS

K. Hleibieh was supported by a Franco-Syrian grant. This research project was carried out with the financial support of the German Science Foundation. We thank D. Scheidecker for technical assistance.

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