Identification of *Beet necrotic yellow vein virus* P25 Pathogenicity Factor–Interacting Sugar Beet Proteins That Represent Putative Virus Targets or Components of Plant Resistance

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Beet necrotic yellow vein virus (BNYVV) induces the most important disease threatening sugar beet. The growth of partially resistant hybrids carrying monogenic dominant resistance genes stabilize yield but are unable to entirely prevent virus infection and replication. P25 is responsible for symptom development and previous studies have shown that recently occurring resistance-breaking isolates possess increased P25 variability. To better understand the viral pathogenicity factor's interplay with plant proteins and to possibly unravel the molecular basis of sugar beet antiviral resistance, P25 was applied in a yeast two-hybrid screen of a resistant sugar beet cDNA library. This screen identified candidate proteins recognized as orthologues from other plant species which are known to be expressed following pathogen infection and involved in plant defense response. Most of the candidates potentially related to host-pathogen interactions were involved in the ubiquitylation process and plants response to stress, and were part of cell and metabolism components. The interaction of several candidate genes with P25 was confirmed in *Nicotiana benthamiana* leaf cells by transient agrobacterium-mediated expression applying bimolecular fluorescence complementation assay. The putative functions of several of the candidates identified support previous findings and present first targets for understanding the BNYVV pathogenicity and antiviral resistance mechanism.

Beet necrotic yellow vein virus (BNYVV) (Tamada and Baba 1973) is the type species of the genus *Benyvirus* accepted by the International Committee on Taxonomy of Viruses (ICTV) in 1997 (Tamada 1999). BNYVV, transmitted by the soilborne plasmodiophoromycete *Polymyxa betae* (Adams et al. 2001; Dessens and Meyer 1996; Keskin 1964; Rush 2003), is the causal agent of rhizomania. Rhizomania is the sugar beet disease with the greatest economic significance because it possesses the ability to severely reduce plant yield (up to 90%) and sugar yield (up to 70%) in susceptible cultivars (Johansson 1985; Richard-Molard 1985; Tamada 1999). For details about the worldwide distribution of rhizomania infested areas, see Gidner and associates (2005) and Richard-Molard and Cariolle (2001).

BNYVV damages sugar beet by the symptoms it induces. BNYVV causes susceptible sugar beet plants to develop an

extensive proliferation of additionally formed but necrotic root hairs which form a root beard. The tap root often reveals a stunted wine glass shape with brownish vasculature. When leaves are invaded, which rarely occurs, leaf veins become yellow and eventually necrotic (Koenig 2008). Little is known about the molecular mechanisms and the virus-induced physiological alterations which form these symptoms. Pollini and Giunchedi (1989) have described the virus-induced histological changes of BNYVV-infested sugar beet root hairs, and Pollini and associates (1990) reported an increase of free auxin exclusively in susceptible infested sugar beet root hairs. Molecular data, supplying an insight into the differential expression of sugar beet root genes following BNYVV infection, were recently generated by Schmidlin and co-workers (2008). Their extensive differential transcriptomic analysis revealed virus- and host-plant-specific expression of candidate genes linked to cell cycle, ubiquitin/proteasome system (UPS), general metabolism, defense signaling, and oxidative stress response. Larson and associates (2008) analyzed proteome changes evaluated with multidimensional liquid chromatography from different sugar beet accessions induced by BNYVV. Results suggested involvement of systemic resistance elements and the role of phytohormones needed for symptom development. The BNYVV genome organization and detailed known functions of virus-encoded proteins are summarized by Koenig (2008). In general, the BNYVV multicomponent genome consists of four, in some isolates five, positive single-stranded RNAs. RNA1 and RNA2 contain all the housekeeping genes necessary for replication, encapsidation, suppression of RNA silencing, and virus movement. RNA4 is involved in vector-mediated transmission and root-specific suppression of RNA silencing (Rahim et al. 2007). RNA3-encoded P25 protein is responsible for rhizomania symptom development in sugar beet and the formation of severe symptoms in leaves of mechanically inoculated sugar beet plants and *Chenopodium quinoa* (Chiba et al. 2008; Klein et al. 2007; Koenig et al. 1991; Tamada 1999, 2007; Tamada et al. 1989). RNA5 (present mainly in Asia and in limited geographic regions in France and the United Kingdom) encodes another pathogenicity factor, P26, involved in symptom expression (Koenig et al. 1997; Link et al. 2005; Miyanishi et al. 1999; Schirmer et al. 2005; Schmidlin et al. 2005; Ward et al. 2007), which may act synergistically with RNA3-encoded P25 (Tamada et al. 1996a).

Due to the severe damage induced by the virus and lack of viable alternative control measures between virus and sugar beet, production is entirely dependant on resistant cultivars to

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control the disease. Lewellen and associates (1987) reported the identification of partially resistant germplasm in field trials of the Holly Sugar Company. Currently, the “Holly” source contains the most important major dominant resistance gene, named *Rz1* (Biancardi et al. 2002; Scholten and Lange 2000). Additionally, wild beet (*Beta vulgaris* subsp. *maritima*) accessions served as sources for further major resistance genes (*Rz2* to *Rz4*) (Gidner et al. 2005; Grimmer et al. 2007; Scholten et al. 1994, 1999; Whitney 1989). The resistance mechanism remains largely unknown, *Rz1*, *Rz2*, and *Rz3* plus a combination of the genetic background and additional minor genes possess the ability to reduce the virus replication and content in hairy roots and restrict virus spread to the tap root (Heijbroek et al. 1999; Luterbacher et al. 2005; Scholten et al. 1994; Tamada et al. 1996b). However, alone or in combination, all known major resistance genes provide only quantitative resistance and do not seem to entirely prevent BNYVV replication and cell-to-cell movement from the initial vector-infected cells in sugar beet root cells.

Several recent publications provided indirect evidence that P25 might be the target of recognition of the sugar beet anti-virus resistance. Even though the different pathotypes A, B, and P of BNYVV possess highly conserved RNA1 and RNA2 components, several studies reported a high variability in the P25 pathogenicity factor amino acid tetrad 67-70, especially in A-type isolates (Chiba et al. 2003; Koenig and Lesemann 2005; Schirmer et al. 2005; Tamada et al. 2003; Ward et al. 2007; Yilmaz et al. 2007). Moreover, this tetrad composition determines lesion type, reflecting resistance response in mechanically inoculated *B. vulgaris* “Rizor” and *B. maritima* leaves, suggesting that P25 functions as an avirulence (*Avr*) gene product (Chiba et al. 2008; Tamada 2007). In addition, Klein and associates (2007) supplied other evidence for the role of P25 amino acid 67-70 tetrad in BNYVV symptom severity in leaves of the host *Tetragonia expansa*. BNYVV P25 represents a nuclear shuttling protein, containing nuclear localization (NLS) and nuclear export (NES) signals (Vetter et al. 2004). Klein and associates (2007) additionally found evidence for P25 oligomerization and an influence of the tetrad composition on this ability.

Recently, BNYVV isolates capable of overcoming *Rz1* resistance in the field have been reported from several independent field locations in the United States and Europe (Liu and Lewellen 2007; Liu et al. 2005; Pferdmenges et al. 2009), indicating that the durability of rhizomania resistance in sugar beet seems to be threatened through the large-scale cultivation of a single resistance source (*Rz1*) (Acosta-Leal et al. 2008).

The objectives of this study were to detect BNYVV cellular targets and to identify resistance factors involved in the recognition of P25 in an incompatible *Rz2*-based interaction. Both interrelations are probably based on physical protein-protein interactions, and their identification and functional analysis of the partners might result in a first understanding of the molecular and cellular mechanisms of this plant-virus interplay. To achieve this, a yeast two-hybrid (YTH) system has proven itself invaluable as a sensitive molecular genetic approach in the mapping of protein-protein interactions in vivo. We have screened for P25-interacting sugar beet proteins by applying a cDNA library from a homozygous *Rz2*-carrying sugar beet line. Many candidate proteins were identified, displaying no detectable transcriptional autoactivation and physical interaction of variable intensity in yeast cells. Subsequent in planta analysis in *Nicotiana benthamiana* leaf cells resulted in proof of expression and interaction, applying a bimolecular fluorescence complementation (BiFC) system based on *Agrobacterium tumefaciens* transient expression.

RESULTS

Identification of sugar beet *Rz2* candidate proteins interacting with BNYVV B-type P25

in yeast cells following a LexA YTH cDNA library screen.

P25 sugar beet proteome interaction of an incompatible host-pathogen interaction (BNYVV B-type versus *Rz2* sugar beet genotype) was studied in a LexA-based YTH system. A cDNA library from a BNYVV B-type infected *Rz2*-harboring sugar beet breeding line, consisting of 1.4×10^6 independent clones, was chosen. In all, 420 green fluorescing colonies were picked out of approximately 1×10^6 transformed yeast colonies and used for further characterization. Deselection of transcriptional autoactivators, retransformation, and repetition of the interaction assay resulted in a total of 36 P25-interacting candidates (Fig. 1), with an insert size varying from approximately 300 to 1,200 bp (Table 1) remaining. The strength of the P25-cDNA encoded candidate interactions was monitored indirectly by quantification of the Leu-driven green fluorescent protein (GFP) expression. GFP expression was detectable in all different cDNA library candidate P25 interaction tests (Fig. 1D); significant differences in strength of the interaction were observed. Nucleotide sequences of the cDNAs were obtained and coding sequences were used for the National Center for Biotechnology Information (NCBI) GenBank database homology BLAST search using BLASTn algorithm. The cDNA sequences were translated and the amino acid sequences additionally applied for a database search making use of the “Simple Modular Architecture Research Tool” (SMART 5) for identification and analysis of domain architectures. The results of these database analyses, including functional categorization and prediction of putative role in the virus-host interaction (i.e., involvement in virus life cycle or antiviral resistance) are summarized in Table 1.

Of 36 cDNA sequences analyzed, 27 cDNA-encoded sugar beet candidate P25-interacting proteins displayed significant (E value $< 1e^{-04}$) homology to plant gene products with known functions. Nine candidate clones did not display significant homology to plant sequences with known functions (pJG4-5-28 to -36). The cDNA inserts of these clones displayed a length of 602 bp (number 28), 454 bp (number 29), 527 bp (number 30), 433 bp (number 31), 497 bp (number 32), 541 bp (number 33), 788 bp (number 34), 452 bp (number 35), and 505 bp (number 36), respectively. SMART database analysis revealed conserved functional or signaling domains for 10 of 27 candidates (candidate numbers 1, 2, 6, 13, 14, 15, 16, 19, 21, and 24). In most cases, the SMART analysis confirmed the results of the BLAST search. The proteins identified in the GenBank BLAST search, showing significant homology to the P25-interacting sugar beet candidates, displayed known or at least putative functions. In accordance with their biological role, all database matches corresponding to candidate numbers 1 to 27 were classified into the following categories: a) UPS; b) stress, pathogen response, and phytohormone signaling; c) cell-structure, cell cycle, and cell trafficking; d) translation and protein modification; e) (general) metabolism; and f) regulators of gene expression. Interestingly, three of 27 candidates, numbers 1 (1,149 bp), 12 (508 bp), and 24 (751 bp), displayed significant homology to plant proteins involved in the UPS, a key regulator for many biological processes in plants. In principle, the system regulates protein stability by covalently attaching ubiquitin to target proteins for 26S proteasome degradation. In addition to phytohormone signaling, light signaling, organ initiation, and patterning, UPS is now known to be essential for plant defense against pathogens. Candidates 1 and 12 are similar to kelch repeat-containing F-box family proteins from *Arabidopsis thaliana* responsible for substrate recognition in the

ubiquitylation process as a member of the E3 ligase complex. Number 24, a candidate with high homology to three different known sequences, is also involved in the ubiquitylation process as a plant U-box protein from *A. thaliana* known as an E3 ligase.

Candidate proteins interacting with P25 in yeast displaying homology to proteins involved in stress and pathogen response as well as phytohormone signaling (candidate numbers 3, 7,

11, 20, and 25) were summarized in one category. Candidate number 3 matched best in GenBank analysis with an ethylene-responsive putative universal stress protein from *A. thaliana*. Leucine-rich repeat (LRR) family proteins or extensin family proteins (*A. thaliana*), to which candidate number 7 possessed highest homology, are essential for cell-wall assembly, and growth by cell extension and expansion and are known to be induced by auxin, biotic, or abiotic stress. Candidate number

Fig. 1. A, Yeast two-hybrid (YTH) interaction assay of *Beet necrotic yellow vein virus* P25 with 36 different *Rz2* sugar beet candidate proteins. P25 was fused to LexA-BD (pEG202-P25) and *Rz2* sugar beet cDNA library clones to B42-AD (pJG4-5-library nos. 1 through 36). The bait and prey combinations, co-transformed into yeast, were selected on medium containing galactose and lacking -Leu, -Trp, -Ura, and -His. **B**, Test for transcriptional autoactivation of the Leu reporter gene: pEG202 co-transformed with pJG4-5-library numbers 1 through 36. **C**, YTH interaction positive control (PC) LTA (pJG4-5-LTA) with P53 (pEG202-P53), negative control (NC), and autoactivation control (both pJG4-5 + pEG202). **D**, Relative quantification of Leu-driven green fluorescent protein fluorescence in yeast cell matings expressing the same P25 and cDNA (numbers 1 through 36) as in A, measured by display total area density of the mean yeast cell fluorescence signal (total of 20 cells) measured in epifluorescence microscopy.

11 revealed significant homology to a plant jasmonate-induced protein with unknown function. An ethylene-responsive element-binding protein with putative DNA binding or transcription factor abilities (*Sesuvium portulacastrum*) resulted from the candidate number 20 GenBank search. Candidate number 25 showed high homology to a putative auxin-responsive gene and transcription factor from *N. tabacum* belonging to the early auxin-responsive Aux/IAA gene family.

From rhizomania root-proliferation symptomatology, it has been deduced that BNYSV influences root hair morphogene-

sis, cell division, and elongation. Therefore, candidates with a putative participation in cell structure, cycle, and trafficking were classified into one category. Candidate number 6 showed high homology to actin (*Carica papaya*) and number 9 to a microtubule-associated protein named TOR1 (*A. thaliana*) which regulates the orientation of cortical microtubules and the direction of organ growth. The protein plays a role in control of microtubule-dependent anisotropic cell elongation. The homologue with the highest expected value to candidate number 10 is predicted to represent a structural component of the

Table 1. National Center for Biotechnology Information (NCBI) GenBank sequences (accession number, function, and conserved domains) displaying significant sequence homology to sugar beet Rz2 candidate cDNA clones identified to show LexA–yeast two-hybrid interaction with *Beet necrotic yellow vein virus* (BNYSV)-encoded P25 pathogenicity factor and prediction of putative candidate function and possible involvement in virus–host interaction

No. ^x	cDNA (bp) ^y	NCBI Blastn ^u			SMART ^v			Invol. ^w		
		Accession no.	Function (organism)	<i>e</i>	Domains	<i>e</i>	Putative function	Cat. ^z	Virus	Resist.
1	1,149	NM_126342.3	Kelch repeat-containing F-box family protein (<i>Arabidopsis thaliana</i>)	1e ⁻⁸⁵	Kelch domains	6.71e ⁻⁰¹ , 1.77e ⁻⁰⁹	Substrate recognition for ubiquitylation	a	x	...
2	533	NM_100310.5	Thioesterase family protein (<i>A. thaliana</i>)	2e ⁻⁵²	Protein phosphatase 2A homologues, catalytic domain.	3.66e ⁻⁰²	Exhibit Esterase activity specifically at a thiol group	d	x	x
3	535	AY589695.1	Putative universal stress protein family protein/ethylene responsive (<i>Mirabilis jalapa</i>)	1e ⁻⁴⁹	...	...	Stress-induced protein	b	...	x
4	535	NM_115492.3	40S ribosomal protein S26 (<i>A. thaliana</i>)	1e ⁻⁴¹	...	...	Ribosomal protein	d	x	...
5	260	NM_180927.3	Protein (serine/threonine) phosphatase 7 (PP7) (<i>A. thaliana</i>)	1e ⁻¹⁰¹	...	...	Regulator of cryptochrome mediated blue light signaling	d	x	x
6	404	AY906938.1	Actin (<i>Carica papaya</i>)	2e ⁻⁶⁹	Actin domain	5.27e ⁻⁰⁵	Structure protein	e	x	...
7	311	NM_103837.1	Leucine rich repeat family protein/extensin family protein (<i>A. thaliana</i>)	5e ⁻¹⁹	...	...	Accumulation in response to wounding, pathogen infection, treatment with elicitor	b	...	x
8	429	DQ335796.1	Cytochrome P450 monooxygenase (<i>Medicago truncatula</i>)	5e ⁻⁰⁸	...	...	Metabolizing compounds	e	...	x
9a	442	AJ507317.1	Potyviral helper component protease-interacting protein 2 (hip2 gene) (<i>Solanum tuberosum</i>)	3e ⁻²²	...	...	Unknown function	c	x	x
9a	...	NM_118840.4	TOR1 (TORTIFOLIA 1) (<i>A. thaliana</i>)	1e ⁻¹³	...	...	Microtubule-associated protein	c	x	x
10	453	NM_112733.2	CCAAT displacement protein-related/ CDP-related (ATCASP) (<i>A. thaliana</i>)	3e ⁻⁸⁷	...	...	Predicted Golgi apparatus structural component	c	x	x
11	571	U15657.1	Jasmonate-induced protein (<i>Atriplex canescens</i>)	1e ⁻⁰⁴	...	...	Homolog of a jasmonate-induced protein from barley, function unknown	b	...	x
12	508	NM_102054.3	Kelch repeat-containing F-box family protein (<i>Arabidopsis thaliana</i>)	5e ⁻³⁶	...	...	Substrate recognition for ubiquitylation	a	x	...
13	349	NM_115171.3	Low expression of osmotically responsive genes 1 (LOS4) (<i>A. thaliana</i>)	2e ⁻²⁵	Helicase superfamily c-terminal domain	1.05e ⁻⁰⁶	Encodes a putative DEAD-box RNA helicase and has RNA-dependent ATPase activity; similar to eukaryotic translation initiation factor 4A	d	x	...

(continued on next page)

^u Accession number of the best matched sequences and predicted, putative, or known functions. Expect (*e*) values of <1e⁻² usually indicate homology and those of <1e⁻⁵ almost always indicate homology (Altschul et al.1997).

^v Simple Modular Architecture Research Tool, showing conserved signaling or functional domains. Expect (*e*) values of <1 usually indicate homology (Schultz et al. 2004). Candidate nos. 1, 15, 16, and 24 are homologue to two or three functional domain repeats; every domain is illustrated by its *e* value.

^w Involvement in virus life cycle and resistance.

^x Division into subclasses (a–c) of GenBank sequences with significant candidate homology but different functions.

^y cDNA length.

^z Functional categories: a) ubiquitin/proteasome system; b) stress and pathogen response, phytohormone signaling; c) cell structure, cell cycle, cell trafficking; d) translation and protein modification; e) metabolism; f) regulator of gene expression; and g) not determined.

Golgi apparatus which is known to be involved in protein modification as well as in synthesis and modification of membranes and cell walls. This cDNA candidate displayed homology to a regulator of gene expression: CCAAT displacement protein-related or CDP-related (ATCASP; defined as “cut alternatively spliced product” from *A. thaliana*). Candidate number 16 displayed homology to fiber annexin and candidate number 27 to a clathrin-binding protein (*A. thaliana*), known to be involved in membrane vesicle formation.

Four candidates were classified into the category “translation and protein modification.” Candidate number 2 revealed high homology to thioesterase proteins (*A. thaliana*), which belong to the class of hydrolases; however, SMART search resulted in the identification of a conserved protein phosphatase domain. In addition, candidate number 5 showed homology to

another *A. thaliana* serine/threonine protein phosphatase. Candidate number 13 revealed high homology to an eukaryotic translation initiation factor (*A. thaliana*).

Following GenBank search, three sugar-beet-encoded proteins isolated within the P25 YTH screen were found to be putatively involved in cell metabolism; namely, number 8, with high homology to a Cytochrome P450 monooxygenase (*Medicago truncatula*); number 23, with a putative involvement in riboflavin synthesis (*A. thaliana*); and number 26, displaying high homology to a triosephosphate isomerase (*Solanum tuberosum*).

In total, four candidates (14, 15, 18, 19, and 22) could be associated with regulation of gene expression. The best match sequence to candidate no. 14 revealed an RNA-recognition motif (*A. thaliana*), which has been found in splicing factors, translation initiation factors, and other RNA-binding proteins.

Table 1. (continued from preceding page)

No. ^x	cDNA (bp) ^y	NCBI Blastn ^u			SMART ^v			Invol. ^w		
		Accession no.	Function (organism)	<i>e</i>	Domains	<i>e</i>	Putative function	Cat. ^z	Virus	Resist.
14	477	NM_101941.2	RNA recognition motif (RRM)-containing protein (<i>A. thaliana</i>)	3e ⁻³⁰	RNA recognition motif	3.43e ⁻²²	Nucleotide-binding motif possibly involved in developmental and signaling pathways	...	...	...
15	560	NM_001111723.1	SET domain-containing protein SET104 (<i>Zea mays</i>)	4e ⁻⁶¹	N-terminal to some SET domains SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain	9.88e ⁻¹⁸ , 7.14e ⁻⁰²	Regulating gene expression through histone methylation	f	x	x
16	564	U89609.1	Fiber annexin (<i>Gossypium hirsutum</i>)	3e ⁻⁹⁴	Annexin repeats	1.34e ⁻¹⁵ , 6.40e ⁻⁰²	Interaction with phospholipid membranes (Ca ²⁺ dependent)	c	...	x
17	307	NM_113308.5	Hydroxyethylthiazole kinase (<i>A. thaliana</i>)	1e ⁻¹⁹	...	...	Thiamine metabolism	g	x	x
18	335	AB040471.1	Bzip transcriptional activator RSG (Repression of shoot growth) (<i>Nicotiana tabacum</i>)	3e ⁻¹⁸	...	...	Transcriptional activator	f	...	x
19	454	NM_102305.1	Zinc finger protein 7 (ZFP7) (C2H2 type) (<i>A. thaliana</i>)	4e ⁻¹⁶	Zinc finger	9.96e ⁻⁰¹	CCCH-type zinc-finger motifs regulate RNA stability	f	x	...
20	388	AY191304.1	Ethylene responsive element binding protein (<i>Sesuvium portulacastrum</i>)	2e ⁻³⁸	...	...	DNA binding	b	...	x
21	578	NM_113083.4	Cyclin-dependent protein kinase (CYCP2.1) (<i>A. thaliana</i>)	2e ⁻⁵³	Domain present in cyclins	1.58e ⁻⁰¹	Cell-cycle progression	c	x	...
22	698	NM_129754.3	Zinc finger (CCCH-type) family protein (<i>A. thaliana</i>)	4e ⁻⁰⁵	...	...	Nucleic acid binding; transcription factor; zinc ion binding	f	x	x
23	589	NM_127633.3	Lumazine-binding protein (<i>A. thaliana</i>)	2e ⁻²²	...	...	Riboflavin synthase	e	x	x
24a	751	EF091878.1	ACRE 276-like protein (<i>Solanum tuberosum</i>)	1e ⁻¹²⁰	Armadillo/β-catenin-like repeats (ARM)	4.03e ⁰¹ , 3.52e ⁰⁰ , 1.08e ⁰⁰	Ubiquitylation of protein substrates	a	...	x
24b	...	AY220483.1	Avr9/Cf-9 rapidly elicited protein 276 (ACRE276) (<i>N. tabacum</i>)	3e ⁻¹⁰⁹	...	...	...	a	...	x
24c	...	NM_102674.3	PUB17 (PLANT U-BOX17); ubiquitin-protein ligase (PUB17) (<i>A. thaliana</i>)	5e ⁻⁷⁴	...	...	...	a	...	x
25	751	AF123508.1	Nt-iaa28 deduced protein (<i>N. tabacum</i>)	3e ⁻¹⁷	...	...	Auxin responsive gene/transcription factor	b	x	...
26	431	DQ200399.1	Triosephosphate isomerase (<i>S. tuberosum</i>)	4e ⁻¹⁰⁶	...	...	Role in glycolysis; essential for efficient energy production	e	x	x
27	417	AK230200.1	Clathrin binding protein (<i>A. thaliana</i>)	5e ⁻⁰⁸	...	...	Cell trafficking	c	...	x

The SET-domain, identified in the SMART search of candidate number 15, is regularly found in methyltransferases (i.e., histone methyltransferases which regulate gene expression (*Zea mays*)). Candidate number 18 displayed homology to a transcriptional activator from *N. tabacum*. Finally, two candidates (numbers 19 and 22) were significant homologues to zinc-finger (C2H2- and CCCH-type, respectively) motifs which can be found in transcription and splicing factors (*A. thaliana*).

P25 interacts with several YTH-identified candidate proteins in living plant cells.

To confirm the interaction of sugar beet candidate proteins with P25, an in planta BiFC with monomeric red fluorescent protein (mRFP1) was selected (Jach et al. 2006; Walter et al. 2004). Two binary vectors for *Agrobacterium*-mediated transient expression were constructed, expressing P25 or a cDNA-encoded candidate fused to the N-terminal 177 amino acids or the C-terminal 66 amino acids of mRFP1, respectively, driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter and polyadenylation signal. To validate the system for its suitability to detect the physical interactions of two proteins, a positive control was developed. *Escherichia coli* β -glucuronidase (GUS) is known to form tetramers (Bracey and Paigen 1987); therefore, it was applied for the in planta BiFC analysis using the newly constructed vectors. Agroinfiltration of mRFP-C-GUS and -N-GUS led to visible reconstitution of mRFP fluorescence at 5 days postinoculation (dpi) (Fig. 2A), whereas infiltration of mRFP-N-GUS plus mRFP-C-empty did not (Fig. 2B), as observed under epifluorescence microscopy (FM). GUS expression was verified in total leaf protein extracts with hemagglutinin epitope (HA) and c-myc specific antibodies at the expected size of 75 kDa (plus the mRFP-C or mRFP-N) (Fig. 3). Subsequently, all 36 sugar-beet-encoded

cDNA candidate genes were cloned into pBin-mRFP-N. The assay was repeated with mRFP-C-P25 plus mRFP-N expressing each of the different (YTH-selected) cDNA candidates. In all, 10 of 36 candidates (namely, candidate numbers 6, 9, 10, 14, 16, 22, 26, 27, 28, and 33) displayed reconstitution of mRFP fluorescence at 5 dpi when co-infiltrated with P25 (Fig. 2C to L). This fluorescence-based detection of in planta interaction was not detected when P25 or the candidate was transiently expressed alone (data not shown). In all cases, detectable mRFP red fluorescence coincided with detection of the candidate-mRFP fusion expression (Fig. 3). Remarkably, the expression level correlated roughly with the strength of the fluorescing signal (e.g., candidate numbers 14, 16, and 22). Strong interactions of cDNA-P25 showed lower fluorescence (i.e., candidate numbers 14 and 16) than the others. All remaining 26 mRFP-N-candidate fusions did not result in detectable mRFP reconstitution when co-infiltrated with mRFP-P25. Out of the candidates where interaction in yeast could not be confirmed, expression of eight candidates (numbers 3, 4, 5, 22, 29, 31, 32, and 33) was detectable in Western blot analysis of total leaf protein extracts following *N. benthamiana* agroinfiltration. Therefore, these were considered to represent additional false-positive P25 interactors and were excluded from further evaluation. Although independent repetitions were performed, the other 18 candidates did not show any expression beyond Western blot detection level.

DISCUSSION

In this study, we aimed to obtain first insights into the virus-host interaction by identifying sugar beet proteins from a resistant genotype showing physical interaction with P25 and to deduce their putative biological role. To our knowledge, this

Fig. 2. Bimolecular fluorescence complementation (BiFC) visualization of *Beet necrotic yellow vein virus* P25 in planta interaction with several *Rz2* sugar beet cDNA-encoded candidate proteins initially identified in yeast two-hybrid library screen. Photos of epifluorescence microscopy ($\times 200$) monomeric red fluorescent protein (mRFP) analysis and bright field microscopy of BiFC assay. *Nicotiana benthamiana* leaf epidermis cells at 5 days postinoculation following agroinfiltration with mRFP-C and -N, fused to P25 and sugar beet candidate interactors, respectively. **A**, Positive control: *Escherichia coli* β -glucuronidase (GUS) tetramerization; **B**, negative control: mRFP-N-GUS plus mRFP-C-empty; **C** through **L**, mRFP-C-P25 plus mRFP-N-cDNA-candidate numbers **C**, 6; **D**, 9; **E**, 10; **F**, 14; **G**, 16; **H**, 22; **I**, 26; **J**, 27; **K**, 28; **L**, 33. Bars in photos **C** and **I** represent a length of 50 nm.

study represents the first systematic YTH cDNA library screen of sugar beet using a viral pathogenicity factor as bait. Although the proof of biological significance of the interactions detected is missing, the data represent a first step toward understanding the cellular life cycle of BNYYV and gives a first indication of how the resistance might control virus replication and systemic spread.

The sugar beet genome consists of 758 Mbp encoding approximately 25,000 genes (Kole 2007). The cDNA library, containing approximately 1.4×10^6 independent clones, resulted in a calculated 18-fold coverage of the sugar beet proteome, which is comparable with cDNA libraries applied in other successful YTH-studies (e.g., *N. tabacum* cDNA library, 1.55×10^6 clones, 15-fold coverage [Soellick et al. 2000]; and *A. thaliana* library, 1×10^6 clones, 13-fold coverage [Pazouhandeh et al. 2006]). The high redundancy might explain the fact that no P25-interacting protein was isolated more than once from the library, although several hundred colonies were picked for further analysis. The deselection process of false-positive candidates with transcription autoactivation ability can be considered to be highly quantitative because it was performed in two repetitions in independent assays using liquid and solid media with different plating densities, respectively, leading to 85% (384/450) deselected candidates. As in the BiFC approach, 18 translational fusions of candidate proteins with mRFP did not lead to detectable transient expression, which might be explained by protein instability; we will not exclude these candidates from evaluation. These 18 BiFC nonexpresser candidates and the remaining 8 candidates did not display in planta BiFC interaction, although being expressed should be used in further studies to confirm the yeast interaction. Remarkably, many of the candidates could be assigned to a function with a plausible relation to the virus life cycle or plant resistance.

The screen revealed three different P25 interactors with high homology to F- and U-box proteins (numbers 1, 12, and 24) placed in category a. Multiple studies have found evidence that mammalian as well as plant viruses try to overcome the stoichiometric imbalance of host proteins over virus proteins by directing their target plant proteins to the 26S proteasome, acting mainly at the point of substrate recognition (Banks 2003). Among plant RNA (and DNA) virus proteins, this function has been identified as an additional mode of action and was demonstrated to be connected to proteins with various

functions, including silencing suppressor proteins (Jin et al. 2007; Pazhouhandeh et al. 2006), movement proteins (Reichel and Beachy 2000), and cell cycle regulators (Aronson et al. 2000). In contrast to virus proteins which mimic an F-box (Aronson et al. 2000; Pazhouhandeh et al. 2006), an F-box motif was not predicted in P25 (data not shown). The biological relevance of the interference of BNYYV P25 with the proteasome remains unknown but a connection of P25's role in enhancing virus replication and spread mediated by influencing the turnover of host proteins seems plausible. Interestingly, upregulation of a sugar beet ubiquitin gene following BNYYV infection was reported in the recent transcriptome analysis of a susceptible genotype (Schmidlin et al. 2008).

Necrotic root hairs and a taproot with brownish vasculature are indicative of a resistance reaction which is unable to limit the virus spread and reflects a stressed tissue status. This is supported by the results of the studies on the effect of BNYYV changes in the sugar beet proteome, where several phytohormone and stress-response-related proteins were identified, including auxin-related proteins and one abscisic-acid-induced protein (Larson et al. 2008). The elevated auxin contents monitored in early studies (Pollini et al. 1990) and the abnormal root hair synthesis and proliferation are probably induced by the virus to increase the number of host cells for the obligate biotrophic *P. betae* vector. Consistently, Schmidlin and associates (2008) detected virus-induced, increased expression of the sugar beet auxin-binding protein 1 gene (*ABP1*). Virus symptoms are dependant on P25 presence; therefore, it was not surprising to find a P25 interaction with proteins related to phytohormone metabolism (category b) such as candidate number 25 with high homology to the Nt-iaa-deduced protein known as an auxin-responsive gene product (Dargeviciute et al. 1998). The authors described that *Aux/IAA*-related proteins play a regulatory role in auxin-induced events during cell elongation. The high homology of candidate number 20 to an ethylene-responsive element-binding protein with key regulatory functions in developmental processes (Jiménez et al. 2005; Leubner-Metzger et al. 1998) suggests another phytohormone-related gene to be a P25 target. The coincidence of candidate number 7 identification with high homology to an LRR-extensin family protein is striking, and in agreement with the findings of Schmidlin and associates (2008), who detected BNYYV-induced upregulation of a sugar beet extensin gene. Extensin is

Fig. 3. Immunodetection of transiently expressed monomeric red fluorescent protein (mRFP)-C-P25 and mRFP-N-candidate fusions tagged with hemagglutinin (HA) and c-myc, respectively, in bimolecular fluorescence complementation assay in total *Nicotiana benthamiana* leaf proteins. **A**, HA-based detection (with specific antiserum) of mRFP-N-candidate fusions (sizes in kDa indicated below the signals) and mRFP-N- β -glucuronidase (GUS)-positive control. **B**, C-myc based detection (with specific antiserum) of mRFP-C-P25- and mRFP-C-GUS-positive control, with the sodium dodecyl sulfate polyacrylamide gel electrophoresis Coomassie-stained loading control shown below.

known to be a key regulatory element of cell-wall development (Cassab 1998) that can be induced by wounding and pathogen infection or abiotic stress (Merkouropoulos and Shirsat 2003; Merkouropoulos et al. 1999; Takemoto et al. 2001). Hence, it seems plausible that P25 targets this protein to suppress a stress response. When we subjected the best matched candidate number 7 sequence (NM_103837.1) to BLAST search (Blastn algorithm; data not shown), another *Arabidopsis* LRR-extensin that regulates root hair morphogenesis and elongation was identified (accession no. NM_101076). Interestingly, null mutants of this allele developed root hairs that frequently abort, swell, or branch (Baumberger et al. 2001), suggesting that P25 might interfere with candidate number 7 to induce virus symptoms. Future studies using the allele from susceptible plants will be necessary to detect a polymorphism and to confirm the biological relevance of this hypothesis.

Consistent with the proteomic study which identified BNYVV upregulation of cell-cycle- and cell-structure-related transcripts and proteins (Larson et al. 2008), we isolated six P25-interacting candidates putatively connected to these processes (category c). Actin (number 6) and fiber annexin (number 16) are components of the cytoskeleton. Members of the actin multigene family are differentially expressed during plant development (Li et al. 2005) whereas plant annexins are involved in membrane processes and represent an important element of the calcium-signaling pathway (Konopka-Postupolska 2007). Annexin was found to function in plant stress response (e.g., osmotic stress) and, due to annexin-actin interaction, a signaling function was proposed (Calvert et al. 1996). Therefore, we suggest the hypothesis that P25 targets these proteins as an additional interference to root hair morphogenesis and cell wall synthesis or, alternatively, influence a possible actin-annexin-mediated signal transduction. Candidate number 27 displayed significant homology to an *Arabidopsis* clathrin-binding protein. Clathrin possesses a key position in cell division and protein trafficking through endocytic pathways and controls formation of membrane internalization (Pérez-Gómez and Moore 2007). Clathrin-coated vesicles have been associated with rapidly growing cells such as root hair tips (Holstein 2002) and this pathway might represent another P25 target for interference with root morphogenesis. In addition to P25 proteasome interference, possible similarities to a *Potyvirus*-silencing suppressor (HC-Pro) are given (Jin et al. 2007). To our surprise, another candidate with homology to a HC-Pro-interacting protein was revealed in our screen. Candidate number 9 showed homology to a HC-Pro-interacting protein (HIP2) with unknown functions previously isolated from potato (Guo et al. 2003). However, the second match of this BLAST search was an *Arabidopsis* protein responsible for cortical microtubules playing an important role in the regulation of the direction of cell expansion (TOR1). Although the virus genera (*Potyvirus* and *Benyvirus*) do not show close phylogenetic relation and their gene expression strategies are divergent, these results suggest a similar mode of action of both pathogenicity factors.

The candidates assigned to the categories d, e, and f show similarity to genes with common functions in translation and protein modification, cell metabolism, and gene expression. Therefore, the biological significance of the P25 interaction for them remains speculative. For example, Larson and associates (2008) also identified proteins induced in primary and secondary metabolism without any associated role in relation to virus–host interactions.

The YTH screen of an R2z sugar beet cDNA library of high quality with the BNYVV-encoded P25, compared with screens with other viral pathogenicity factors (Anandalakshmi et al. 2000; Guo et al. 2003; Jin et al. 2007; Pazhouhandeh et al. 2006), uncovered an outstanding number of candidates possibly

involved in virus pathogenicity, virus life-cycle, or plant resistance response in sugar beet. There is no doubt that additional interaction tests with each of the candidates, where BiFC assay failed, are necessary to prove these interactions. With high probability, isolation of full-length sequences from susceptible and resistant genotypes for sequence comparison and YTH repetition as well as genomic mapping will reveal sugar beet antiviral resistance factors leading to a better understanding of this plant–virus interaction.

MATERIALS AND METHODS

YTH system, yeast strains, plasmids, and medium.

Saccharomyces cerevisiae strains EGY48 (MAT α , *trp1 his3 ura3 leu2::6 LexAop-LEU2*) (Estojak et al. 1995) and RHY206 (MAT α *trp1D::hisG his3D200 ura3-52 lys2D201 leu2-3*) were obtained from MoBiTec GmbH (Göttingen, Germany) and Origene Technologies Inc. (Rockville, MD., U.S.A.), respectively. Yeast full media, selective drop-out media, and amino acid stock solutions (YPD and YNB-DOB) supplemented with glucose or galactose (glu/gal) were prepared using standard protocols (Origene *DupLEX-A* User manual). Mobitec Grow 'n' Glow GFP Two-Hybrid System (MoBiTec GmbH) is a modified version of the LexA/B42 YTH system originally developed by Fields and Song (1989). It consists of the bait plasmid pEG202, prey plasmid pJG4-5 (Gyuris et al. 1993), and reporter plasmid pGNG1 (Cormack et al. 1998) containing the GFPuv variant (Cramer et al. 1996) under control of four copies of the *lexA* operators within a minimal *GAL1,10* promoter. Plasmids were propagated in *E. coli* DH5 α .

Molecular biology techniques.

All plasmid manipulations were performed according to standard techniques (Sambrook et al. 1989) unless otherwise specified. Manipulations were verified through sequencing (MWG Biotech AG, Martinsried, Germany).

Cloning of the P25 open reading frame into pEG202 YTH bait plasmid.

Plants of a BNYVV-susceptible sugar beet hybrid (cv. Tatjana, supplied by KWS, Einbeck, Germany) were grown in a German field soil sample infected with rhizomania pathotype B (soil origin: Büttelborn) for 4 weeks in the greenhouse. Total RNA (Hamilton et al. 2002) was prepared from (BNYVV-infected) lateral roots, and the entire BNYVV P25 of RNA3 encoded open reading frame (ORF) (nt. 447-1103 in Genbank accession number M36894) was reverse-transcription polymerase chain reaction (RT-PCR) amplified with specific primers P25-up (5'-TTGAATTC ATGGGTGATATATTAGGCG-3', restriction sites in italics and viral nucleotides underlined) and P25-low (3'-TACCTCGAGCTAATCATCATCAACAC-5') introducing restriction sites *EcoRI* and *XhoI* and subsequently cloned into the plasmid pEG202 to obtain a translational fusion of P25 with the LexA DNA binding domain (BD). The B-type P25 ORF insert was verified through sequencing, revealing a composition of the amino acid tetrad position 67-70 of AYHR.

Construction of a sugar beet cDNA library.

Plants of a homozygous sugar beet breeding line (8T_0015, supplied by KWS, Einbeck, Germany) carrying the R2z BNYVV resistance gene were grown in the above-described BNYVV B-type-infested soil for 4 weeks. Root and leaf tissue was harvested and used together for preparation of 2 μ g of total RNA. Generation of the cDNA library in pJG4-5 was carried out by "vertis" Biotechnology AG (Freising-Weihenstephan, Germany). The cDNA synthesis was carried out with

Moloney murine leukemia virus reverse transcriptase and a random linker primer containing an *XhoI* restriction site. Synthesis of the second strand was achieved with a second linker primer containing *EcoRI* (5'-GAATTCATCAGGCAGAGGACGAGACAGAACGCA-3', restriction site in italics) and Klenow polymerase. After PCR amplification, normalization, and size fractionation of cDNAs greater than 0.4 kb, the library was *EcoRI-XhoI* cloned into pJG4-5 to obtain a translational fusion of the cDNAs with the activation domain (AD) B42 and HA-tag. In total, the library contained a number of 1.4×10^6 recombinant clones.

Yeast transformation and YTH sugar beet R_{z2} cDNA library interaction screen with BNYVV P25 applying quantitative liquid mating.

The pJG4-5 cloned sugar beet cDNA library (1.4×10^6 independent clones) was quantitatively transformed into yeast strain EGY48 (MAT α) following the "High-Efficiency Transformation Protocol" of Gietz and Woods (2002) with slight modifications. As carrier DNA, denatured Herring Testes DNA (Clontech, Palo Alto, CA, U.S.A.) (10 mg/ml) was used, the incubation time was increased to 1 h, and centrifugation was carried out at $3,000 \times g$. Approximately 5.2×10^8 colonies growing on selective medium (YNB-DOB-(glu)-trp) were propagated, pooled, and stored in aliquots at -70°C , following the method described by Soellick and Uhrig (2001). In all, 50% of the cells in thawed aliquots were determined to be viable. pEG202-P25 and pGNG1 were co-transformed into RHY206 (MAT α) selected on YNB-DOB-(glu)-ura-his and tested for transcriptional autoactivation of LexA-controlled (pGNG1-encoded) GFPuv and (genome-integrated) LEU2 reporter genes. The empty plasmid pJG4-5 was supertransformed into RHY206 (pEG202-P25, pGNG1) and retested for autoactivation (YNB-DOB-(glu)-ura-his-trp). Quantitative liquid mating under optimized conditions of freshly grown RHY206 (pEG202-P25, pGNG1) with a melted long-term culture aliquot of EGY48 (pJG4-5-cDNA-library) was carried out as described by Soellick and Uhrig (2001) and double-transformed zygotes were selected (YNB-DOB-(gal)-ura-his-trp-leu). Both cultures were mixed for mating at a Mata:Mata α ratio of 2.5:1 with a cell density of 6×10^8 (ratio of bait/GFP cells, 3.75×10^8 to prey cells, approximately 1.5×10^8). The mating reaction was plated (YNB-DOB-(gal)-ura-his-trp-leu) at a density of 2×10^5 cells cm^{-2} , resulting in a mating efficiency calculated by numbers of zygotes in ratio to total cells (including dead cells) of 11%. The number of double transformants was calculated as 7×10^8 . GFP-fluorescing colonies ($n = 450$) were identified with a hand-held long-wave ultraviolet (UV) lamp (Blak Ray model B 100 AP, 100 W; UV Products Upland, CA, U.S.A.), restreaked on solid selective media, and tested for autoactivation in liquid medium (YNB-DOB-(glu)-ura-his-trp-leu) to deselect transcriptional autoactivator cDNAs. In all, 37 candidate interactors with no detectable autoactivation activity were applied for further analysis. Plasmid DNA from yeast was recovered using the protocol described in the Origene DupLEX-A User manual. To selectively recover only pJG4-5 cDNA library plasmids, each isolated yeast plasmid DNA was transformed into *E. coli* strain KC8 (*pyrF*, *leuB600*, *trpC*, and *hisB463*) auxotrophic for tryptophan and reisolated on minimal media (M9) (Sambrook and Russell 2001) lacking tryptophan and supplemented with ampicillin. All candidate pJG4-5 cDNA library plasmids were retransformed into EGY48 and liquid mated with RHY206 (pGNG1+pEG202-P25) following the method described (Yeast Protocols Handbook 2008; Clontech Laboratories Inc.) For each interaction mating of a candidate with P25, a test for autoactivation was performed by mating with RHY206

(pGNG1+pEG202-empty). Then, 5 μl of the dilutions series (10^0 to 10^{-5}) of the mating reaction was spotted on selective medium. At 5 to 7 days after incubation, both matings were checked for interaction and autoactivation, respectively. Only candidates displaying yeast growth at 100-fold higher dilution than the autoactivation control were considered to be nontranscriptional autoactivators. In all YTH interaction assays, appropriate positive controls consisting of mouse P53 (Iwabuchi et al. 1993) fused with DNA-BD (pEG202-P53) and LTA fused with DNA-AD (pJG4-5-LTA) were included (Li and Fields 1993). Total area optical density analysis of GFP fluorescence was carried out using epifluorescence microscopy of yeast cells carried out with Gel-Pro analyzer software 6.0 (Media Cybernetics, Silver Springs, MD, U.S.A.).

Total yeast and plant protein extraction, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electroblot.

Proteins were extracted from yeast cells following the method of Horvath and Riezman (1994) with modified extraction and sodium dodecyl sulfate (SDS) sample buffer (0.02 M Tris, pH 8.0; 0.2 mM EDTA; 2% SDS; 10% mercaptoethanol; 20% glycerol; and 0.05% [wt/vol] bromophenolblue).

Extraction of total plant proteins was carried out by grinding 100 mg of agroinfiltrated *N. benthamiana* leaf tissue in liquid nitrogen. The frozen leaf powder was transferred into a 1.5-ml tube and filled with 500 μl of extraction buffer (95°C). The homogenized sample was boiled for 5 min and centrifuged for 5 min at $11,000 \times g$. The protein samples (20 μl) were separated by 15% SDS polyacrylamide gel electrophoresis and electroblotted on nitrocellulose (GE Healthcare Europe, Freiburg, Germany) for LexA-detection or polyvinylidene difluoride (Roche, Mannheim, Germany) membranes for HA and c-myc detection.

Immunodetection of HA-, LexA-, and c-myc-tagged proteins.

After blocking the membrane with 2% (HA and c-myc) or 1% (LexA) skimmed milk, immunodetection of HA was carried out using anti-HA high-affinity rat monoclonal antibody (Roche 11 867 423 001, 1/800) and alkaline phosphatase (AP)-conjugated goat anti-rat immunoglobulin G (IgG) (whole molecule) (Sigma A9654, 1/7,000 [Sigma, St. Louis]). LexA was detected with polyclonal rabbit anti-LexA (Invitrogen 46-0710, 1/5,000 [Invitrogen, Karlsruhe, Germany]) and AP-conjugated goat anti-rabbit polyclonal antibody (Sigma A3687, 1/10,000). C-myc was probed with anti-c-myc mouse monoclonal IgG (Roche 11 667 149 001, 1/500) and AP-conjugated goat anti-mouse IgG (BioRad 170-6520, 1/500 [Bio-Rad, Hercules, CA, U.S.A.]). Detection was performed using chromogenic substrates 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride reagents (Applichem, Darmstadt, Germany).

Sequence analysis.

The entire cDNA inserts of library-derived candidate genes were determined by a commercial company (MWG Biotech AG, Martinsried, Germany) with standard primers flanking the multiple cloning site of pJG4-5 and applied for database comparison using the nucleotide blast program with the Blastn algorithm within the basic local alignment search tool (BLAST) using the NCBI database (Altschul et al. 1997). The search was limited to sequences from the taxa Viridiplantae (taxid:33090).

cDNA sequences were translated with VectorNTI software (Invitrogen) and applied for the Web-based SMART 5 analysis to identify functional and signaling domains within the sugar

beet protein sequences (Letunic et al. 2006; Schultz et al. 1998).

In planta BiFC.

The red BiFC system based on improved monomeric red fluorescent protein (mRFP1-Q66T) (Jach et al. 2006) was adapted to the specific needs of the library clones. The coding sequence of mRFP1-Q66T mutant was provided by J. Uhrig (University of Cologne, Germany). In accordance with the work of Jach and associates (2006), the coding sequences for the N-terminal amino acids 1 to 168 and C-terminal amino acids 169 to 225 were PCR amplified using specific primers. Between mRFP-N and P25 and between mRFP-C and the sugar beet coding region, a c-myc tag and an HA-tag, respectively, was inserted by attaching the coding sequence to the lower mRFP-N and -C primers. Binary vector pBIN19 (Bevan 1984) was supplied with an enhanced CaMV 35S promoter and polyA terminator cassette (supplied by E. Maiss, Leibniz University, Hannover, Germany) (pBIN19-35S-pA). The mRFP-N-c-myc and mRFP-C-HA PCR products were cloned into pBIN19-35S-pA, leading to pBIN19-mRFP-N-c-myc and -C-HA. Primer sequences and plasmids are available on request.

The BNYVV P25 ORF was *EcoRI-XhoI* cloned into pBIN19-mRFP-N-c-myc. cDNAs from pJG4-5 clones displaying P25 interaction in yeast but no transcriptional autoactivation were cloned similarly into pBIN19-mRFP-C-HA. To obtain a BiFC-positive control, the *E. coli* GUS ORF (Vancanneyt et al. 1990) was PCR reamplified from a GUS plant expression vector (pTQ-GUS-N, supplied by E. Maiss) with specific primers introducing *EcoRI* and *XhoI* restriction sites to obtain a translational N-terminal fusion with mRFP-N and mRFP-C for use in BiFC. Cloned sequences were verified by sequencing.

Agrobacterium-mediated transient expression BiFC assay.

Binary vectors were electrotransformed into *Agrobacterium tumefaciens* strain C58C1 (pGV2260). *A. tumefaciens* cultures were prepared for agroinfiltration essentially as described (Voinnet et al. 2003) with an optical density at 600 nm (OD₆₀₀) adjusted to 1.0. To suppress RNA silencing, a potyviral HC-Pro (35S-HC-Pro) (Varrelmann et al. 2007) of *Plum pox virus* (PPV) was coexpressed by *A. tumefaciens* at an OD₆₀₀ of 1.0 diluted (1:50). For transient expression assay, leaves of 4-week-old *N. benthamiana* plants were co-infiltrated together with the helper component of PPV into epidermis cells of *N. benthamiana* and leaf patches were examined for mRFP fluorescence using FM at 3 to 5 dpi. pBIN-mRFP-N-GUS and pBIN-mRFP-C-cDNA were negative controls and pBIN-mRFP-N-GUS and pBIN-mRFP-C-GUS were positive controls. mRFP fluorescence was detected with FM (Leica DMR) using specific DsRed filters (Emitter HQ 620/60, Beamsplitter Q 585 LP, Exciter HQ 565/30). Photographs were taken using a Leica DFC camera, DFC300 FX.

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NCBI database: www.ncbi.nlm.nih.gov/blast
 SMART database: smart.embl-heidelberg.de