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# Massive up-regulation of LBD transcription factors and EXPANSINs highlights the regulatory programs of rhizomania disease

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## SUMMARY

Rhizomania of sugar beet, caused by *Beet necrotic yellow vein virus* (BNYVV), is characterized by excessive lateral root (LR) formation leading to dramatic reduction of taproot weight and massive yield losses. LR formation represents a developmental process tightly controlled by auxin signaling through AUX/IAA-ARF responsive module and LATERAL ORGAN BOUNDARIES DOMAIN (LBD) transcriptional network. Several LBD transcription factors play central roles in auxin-regulated LR development and act upstream of EXPANSINS (EXPs), cell wall (CW)-loosening proteins involved in plant development via disruption of the extracellular matrix for CW relaxation and expansion. Here, we present evidence that BNYVV hijacks these auxin-regulated pathways resulting in formation LR and root hairs (RH). We identified an AUX/IAA protein (BvAUX28) as interacting with P25, a viral virulence factor. Mutational analysis indicated that P25 interacts with domains I and II of BvAUX28. Subcellular localization of co-expressed P25 and BvAUX28 showed that P25 inhibits BvAUX28 nuclear localization. Moreover, root-specific *LBDs* and *EXPs* were greatly upregulated during rhizomania development. Based on these data, we present a model in which BNYVV P25 protein mimics action of auxin by removing BvAUX28 transcriptional repressor, leading to activation of *LBDs* and *EXPs*. Thus, the evidence highlights two pathways operating in parallel and leading to uncontrolled formation of LRs and RHs, the main manifestation of the rhizomania syndrome.

**Keywords:** beet necrotic yellow vein virus, rhizomania, expansin, P25 virulence factor.

## INTRODUCTION

Plant viruses continue to pose a significant and constant threat to crop production affecting the yield and quality of harvested tissues. Crop losses to viruses compromise global food security and, in many cases, are attributed to virus-reprogrammed plant morphogenesis and abnormal growth, manifested as viral disease symptoms. However, with a few exceptions, the molecular basis of symptom development in plants is still poorly understood (Jin *et al.*, 2016; Lukhovitskaya *et al.*, 2013; Shimura *et al.*, 2011). Rhizomania of sugar beet, caused by *Beet necrotic yellow vein virus* (BNYVV), is characterized by the abnormal proliferation of lateral roots (LRs) leading to a significant decrease in taproot weight, sugar content and massive yield losses. Hence, BNYVV is able to alter the morphogenesis of the sugar beet crop to facilitate severe and dramatic changes in plant development manifested as excessive formation of LRs and root hairs (RHs).

LR emergence and formation represent a tightly controlled developmental process (Péret *et al.*, 2009). The phytohormone auxin and several of its transport and signalling components play a crucial role during LR development (reviewed in Lavenus *et al.*, 2013). When pumped into the cell by specialized transport machinery, the auxin signal triggers the degradation of AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins through binding to the $SCF^{TIR1}$ multiprotein complex (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005; Tan *et al.*, 2007). AUX/IAA proteins function as transcriptional repressors and their degradation allows interacting transcriptional activators, termed AUXIN RESPONSE FACTORs (ARFs), to alter auxin-responsive gene expression (Dharmasiri and Estelle, 2004). In *Arabidopsis thaliana*, two transcriptional repressors, IAA14 and IAA28, have been demonstrated to play an essential role in LR formation (reviewed in Lavenus *et al.*, 2013). Both IAA14 and IAA28 are capable of interacting with ARF7 and ARF19 transcription factors (TFs) (De Rybel *et al.*, 2010; Fukaki *et al.*, 2005). Moreover, the degradation of IAA14 allows ARF7 and ARF19 to activate the

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expression of auxin-responsive genes, including LATERAL ORGAN BOUNDARIES DOMAIN (LBD) TFs. Three LBDs, LBD16, LBD18 and LBD29, play crucial and distinct roles in auxin-regulated LR development (reviewed in Lavenus *et al.*, 2013). LBD16 is involved in instigating the migration of nuclei and in the asymmetric division of LR founder cells to promote LR initiation (Goh *et al.*, 2012). LBD18 plays a role in LR initiation and emergence (reviewed in Lavenus *et al.*, 2013). LBD18 facilitates LR initiation through the transcriptional activation of a cell cycle regulator, E2Fa TF (Berckmans *et al.*, 2011). LBD18 induces the expression of other cell cycle regulators, such as CYCLINB1;1 and CYCLIN-DEPENDENT KINASE A1;1 (Lee *et al.*, 2015). At later stages of LR emergence, LBD18 activates *EXPANSIN A14* (*EXPA14*) expression by directly binding to the *EXPA14* promoter, and indirectly up-regulates *EXPA17* and other *EXPANSIN*s (*EXP*s) to mediate cell wall (CW) loosening and relaxation for cell growth and extension (Lee *et al.*, 2013; Lee *et al.*, 2015).

EXPs are non-hydrolytic CW-loosening proteins involved in the control of cell extension and are engaged in a variety of plant developmental processes, including leaf emergence, LR formation and RH elongation (reviewed in Cosgrove, 2015). In flowering plants, EXPs are encoded by a large multigene family comprising 29–88 members, and are phylogenetically classified into four groups, designated as *EXPANSIN A* (*EXPA*), *EXPANSIN B* (*EXPB*), *EXPANSIN-LIKE A* (*EXLA*) and *EXPANSIN-LIKE B* (*EXLB*) (Kende *et al.*, 2004). The expression of most root-specific *EXP*s is regulated by auxin and promoters of these genes often contain multiple auxin-responsive factor binding elements (ARFEs) and/or conserved RH-specific *cis*-elements (RHEs) (Kim *et al.*, 2006). Two RH-specific *A. thaliana* genes, *AtEXPA7* and *AtEXPA18*, and their functional equivalents in other species, contain RHE sequences in their promoters and are expressed specifically in RH cells immediately prior to RH initiation and elongation (Kim *et al.*, 2006).

BNYVV is vectored in soil by zoospores of the obligate biotrophic parasite *Polymyxa betae*, a ubiquitous plasmodiophorid. Because BNYVV has a world-wide geographical distribution, in sugar beet-growing areas, the growth of BNYVV-resistant sugar beet varieties is essential to maintain high yields. Modern varieties contain the *Rz1* resistance gene (Biancardi *et al.*, 2002), which does not provide complete resistance to infection. However, it delays the viral spread from infected LRs to the main taproot. Two different monogenic resistance genes, *Rz2* and *Rz3*, have been identified in a sea beet: *Beta vulgaris* ssp. *maritima* (Gidner *et al.*, 2005; Lewellen *et al.*, 1987). Recently, the candidate gene for *Rz2* has been identified by mapping-by-sequencing and appears to represent a typical plant R gene encoding a coiled-coil nucleotide-binding leucine-rich repeat (CC-NB-LRR) protein (Capistrano-Gossmann *et al.*, 2017).

BNYVV has a multipartite genome consisting of four or five (depending on the isolate) positive-sense, single-stranded RNA segments (Bouzoubaa *et al.*, 1986; Tamada *et al.*, 1999). RNA1 and RNA2 encode genes of the 'housekeeping module' involved in virus replication, cell-to-cell movement, transmission and encapsidation, whereas the *P14* gene encoded by RNA2, as well as genes encoded by RNA3, RNA4 and RNA5, represent the 'interactive module' comprising genes involved in virus–host interactions (Bouzoubaa *et al.*, 1986; Tamada *et al.*, 1999). The BNYVV RNA3-encoded P25 protein is responsible for rhizomania symptom development in sugar beet (reviewed in Peltier *et al.*, 2011). A short sequence of the P25 gene coding for four consecutive amino acid residues (amino acids 67–70)—the so-called 'tetrad'—shows great variability (Bornemann *et al.*, 2015; Chiba *et al.*, 2011; Schirmer *et al.*, 2005). BNYVV strains with certain 'tetrad' variants harbouring specific mutations are able to overcome *Rz1* and induce typical rhizomania symptoms (Acosta-Leal *et al.*, 2010; Koenig *et al.*, 2009).

BNYVV RNA3 must be present in the inoculum to facilitate virus long-distance movement in *Beta* species (Lauber *et al.*, 1998). The RNA3-encoded P25 protein is a virulence factor of the virus responsible for the 'hairy root' phenotype, when expressed on its own in transgenic *A. thaliana* (Peltier *et al.*, 2011). At the subcellular level, the P25 protein localizes to the nucleus and to the cytoplasm (Vetter *et al.*, 2004). The nuclear localization signal (NLS), zinc-finger domain and nuclear export signal (NES) have been identified in P25 and characterized by mutagenesis (Vetter *et al.*, 2004).

Previous studies in *B. vulgaris* and *Arabidopsis* on the identification of differentially expressed transcripts in response to infection or ectopic BNYVV P25 expression have suggested the importance of EXPs in rhizomania symptom development (Peltier *et al.*, 2011; Schmidlin *et al.*, 2008). Here, we show that, among 32 *EXP* genes of sugar beet, 13 were activated during rhizomania development. Moreover, three LBD TFs that act upstream of the root-specific EXPs in model plant species, but downstream of auxin signalling and the AUX-ARF7/19-responsive module, were also found to be up-regulated. Furthermore, we identified BvAUX28 as physically interacting with P25. Our results indicate that the activation of LBD TFs, and subsequently root-specific EXPs, plays an integral role in shaping the development of the sugar beet root and suggests a link between the dysregulation of BvAUX28 and uncontrolled LR emergence. Taken together, these results support the notion that BNYVV P25 mimics the action of auxin in the induction of LRs and RHs, the phenotypic manifestations of rhizomania disease.

## RESULTS

### Bioinformatics analysis identifies 32 *EXP* genes in the genome of *B. vulgaris*

Previous studies on the identification of differentially expressed transcripts in response to infection or expression of BNYVV P25 have suggested the importance of EXPs in rhizomania symptom

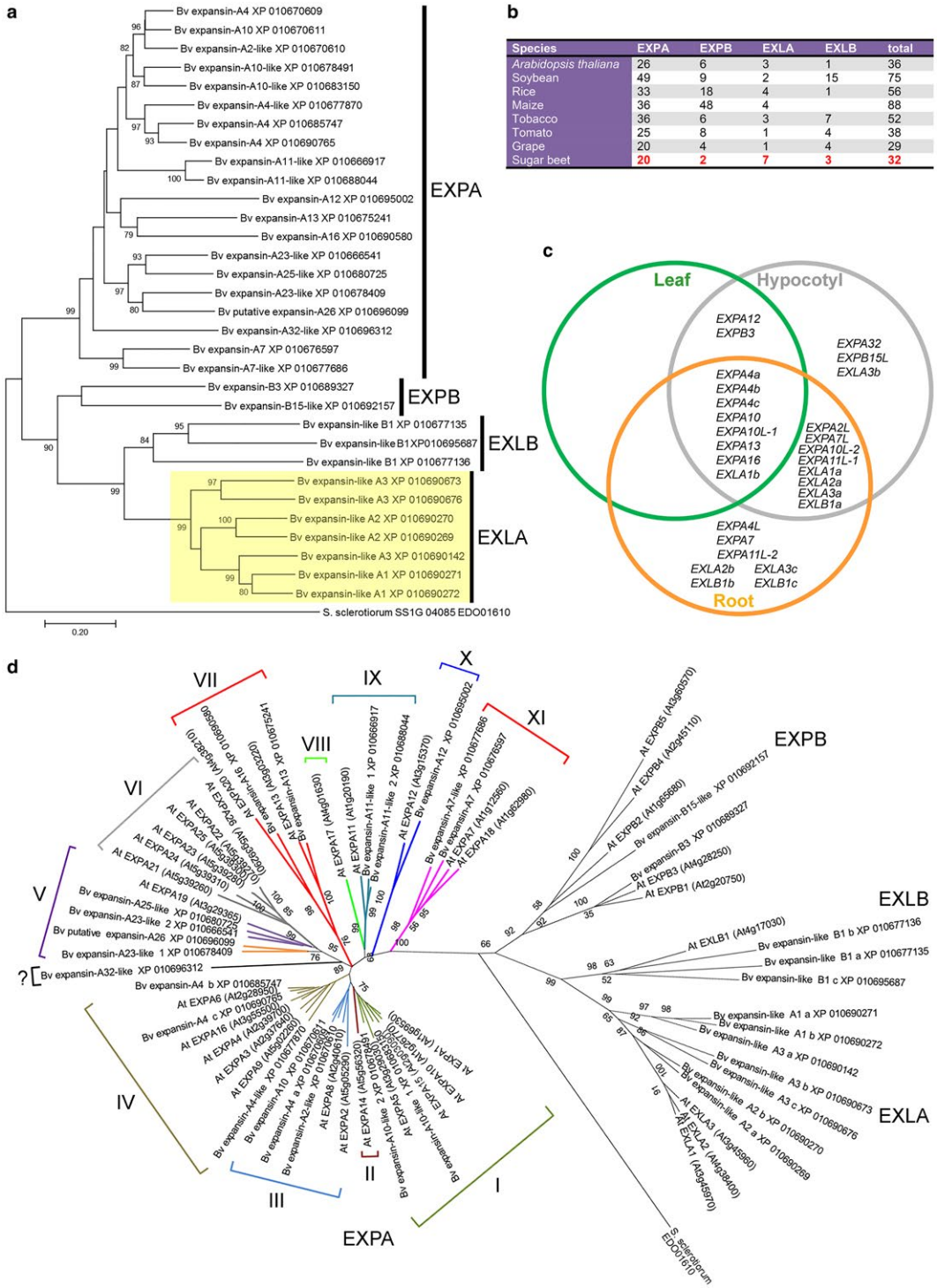


**Fig. 1** Expansin gene family in *Beta vulgaris*. (a) Phylogenetic analysis of the EXPANSIN (EXP) family in *B. vulgaris*. EXP of *Sclerotinia sclerotiorum* was used as outgroup. (b) Minimum gene numbers reported for the four *EXP* subfamilies of representative plant genomes compared with those in *B. vulgaris*. (c) Expression of *EXP* genes in root, hypocotyl and leaf of *B. vulgaris*. Data in the Venn diagram are compiled from our reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results shown in their entirety in Table S2 (see Supporting Information). (d) A phylogenetic tree of the EXP family, including protein sequences from *Arabidopsis thaliana* and *B. vulgaris*. This tree does not correctly resolve clade EXPA-V, possibly because of changes in amino acid usage between Arabidopsis and sugar beet EXPs. The clades of the EXPA subfamily are numbered according to a previous classification in Sampedro and Cosgrove (2005). [Colour figure can be viewed at wileyonlinelibrary.com]

development (Peltier *et al.*, 2011; Schmidlin *et al.*, 2011). As a result of unknown orthology relations, it was unclear which *EXP*s might be up-regulated in *B. vulgaris.* Moreover, different EXPs may have a similar function (functional equivalents) in various plant species. The sugar beet *EXP* gene family is represented by many members. Despite the fact that the *B. vulgaris* genome was sequenced over 4 years ago (Dohm *et al.*, 2014), to the best of our knowledge, there have been no systematic studies on the *EXP* gene family. Because of this gap, a genome-wide analysis of *B. vulgaris EXP*s is required. To this end, using the *Arabidopsis EXP*s as queries, we identified a total of 32 putative *B. vulgaris EXP* gene sequences (Table S1, see Supporting Information).

To investigate the evolutionary relationships of *B. vulgaris EXP*s, we performed multiple sequence alignment and phylogenetic analysis. The results showed that the *B. vulgaris* genes were clearly divided into two large subfamilies, *EXPA* and *EXPB*, and two smaller subfamilies, *EXLA* and *EXLB* (Fig. 1a). This clustering confirmed previous classification of *EXP*s in *Arabidopsis*, rice and other plant species (Cosgrove, 2015). Notably, compared with other plant species, the *EXLA* subfamily is expanded in *B. vulgaris* to seven members (Fig. 1a,b). A previous analysis of the 26 *EXPA* gene sequences in *Arabidopsis* allowed their classification into a total of 12 clades (Sampedro and Cosgrove, 2005). To define orthologous groups, we performed multiple sequence alignment and phylogenetic analysis by comparing the 32 *EXP* gene sequences of sugar beet and the 36 *EXP* gene sequences of *Arabidopsis*. Most *BvEXPA* genes clustered together with the corresponding *Arabidopsis* genes into ten defined clades (Fig. 1d). Notably, two small clades—clades II and VIII—as well as clade VI were exclusively represented by *Arabidopsis* genes, of which *AtEXPA14* and *AtEXPA17* are involved in LR development (Lee and Kim, 2013). The lack of *AtEXPA14* and *AtEXPA17* orthologues in the sugar beet genome suggests that their function in LR development is taken over by other *EXP*s.

To characterize the pattern of *EXP* gene expression, roots and hypocotyl samples were collected from 3-week-old healthy seedlings and from young fully expanded leaves of 4-week-old healthy *B. vulgaris* plants. RNA was extracted and the abundance of individual *EXP* transcripts was assayed by reverse transcription-quantitative polymerase chain reactions (RT-qPCRs) (Table S2, see Supporting Information). The RT-qPCR data indicated that a total of 23 *EXP* genes are expressed to some extent in *B. vulgaris* root tissues (Fig. 1c).

## Identification of *EXP* genes induced in response to rhizomania development

To identify *EXP* genes and investigate the abundance change of their transcript levels in response to rhizomania development, seedlings of a BNYVV-susceptible sugar beet variety were grown in different soils infested with BNYVV and root samples were collected for RNA extraction from the infected and mock-inoculated plants at two time points: 4 and 6 weeks post-sowing (wps). The soils used in these experiments were collected from three locations in Europe [Germany, Sweden and the Netherlands (Holland)], and were characterized previously as containing the B-strain of BNYVV (Germany and Sweden) and the A-strain of BNYVV (Holland) (Koenig and Lennefors, 2000; Lennefors *et al.*, 2008). The presence of BNYVV and *P. betae* in the sampled roots of the plants grown in infested soils was confirmed by RT-PCR and sequencing (Fig. S1a,b,d, see Supporting Information).

To determine which *EXP*s showed altered expression in response to rhizomania development, RT-qPCRs were conducted for 26 *EXP* genes using total RNAs of mock-inoculated and virus-infected plants. The mRNA levels of 13 *EXP*s were significantly higher (Student's two-tailed *t*-test, $P < 0.05$) in virus-infected relative to mock-inoculated plants at 4 wps (Fig. S2a, Table S3, see Supporting Information). A similar set of *EXP*s was up-regulated at 6 wps, but the levels of induction were, depending on the gene, either higher or lower compared with the 4-wps time point, and mRNA levels for three *EXP*s (*BvEXPA7L*, *BvEXPA13* and *BvEXLA2a*) were lower than those in mock-inoculated plants. Collectively, these data suggest highly dynamic changes in *EXP* expression during rhizomania development.

## Three LBD TFs are up-regulated by natural soil-mediated infection with BNYVV

The expression of at least three expansins (*AtEXPA4*, *AtEXPA14* and *AtEXPA17*), which mediate the loosening and remodelling of CW during LR development in *Arabidopsis*, is induced directly or indirectly by AtLBD16, AtLBD18 and AtLBD29. To determine whether natural soil-mediated infection with BNYVV also affects *BvLBD16*, *BvLBD18* and *BvLBD29* expression, RT-qPCRs were conducted. In infected plants displaying typical rhizomania symptoms, the mRNA levels of *BvLBD16*, *BvLBD18* and *BvLBD29* were significantly higher than those in the roots of healthy plants, as measured at two time points (Fig. S2b). Similar to previous observations with *EXP*s, the induction of the *LBD* TFs during rhizomania disease development was highly dynamic with mRNA expression levels noticeably higher at the early stage of virus infection (4 wps) relative to a later sampling date (6 wps) (Fig. S2b). These results indicate that the expression of *LBD* TFs, presumably involved in LR formation in *B. vulgaris* (Table S4, see Supporting Information), is indeed up-regulated by soil-mediated infection.

## Identification of *EXP*s and *LBD* TFs specifically involved in rhizomania development through the use of *Rz1* and *Rz2* resistant varieties, benyvirus infectious clones and virus reassortants

We identified 13 *EXP*s and three *LBD*s as being activated in response to rhizomania development by growing plants in BNYVV-infested soils. In principle, some of these *EXP*s/*LBD*s may be induced by *P. betae* and/or by other unidentified pathogens

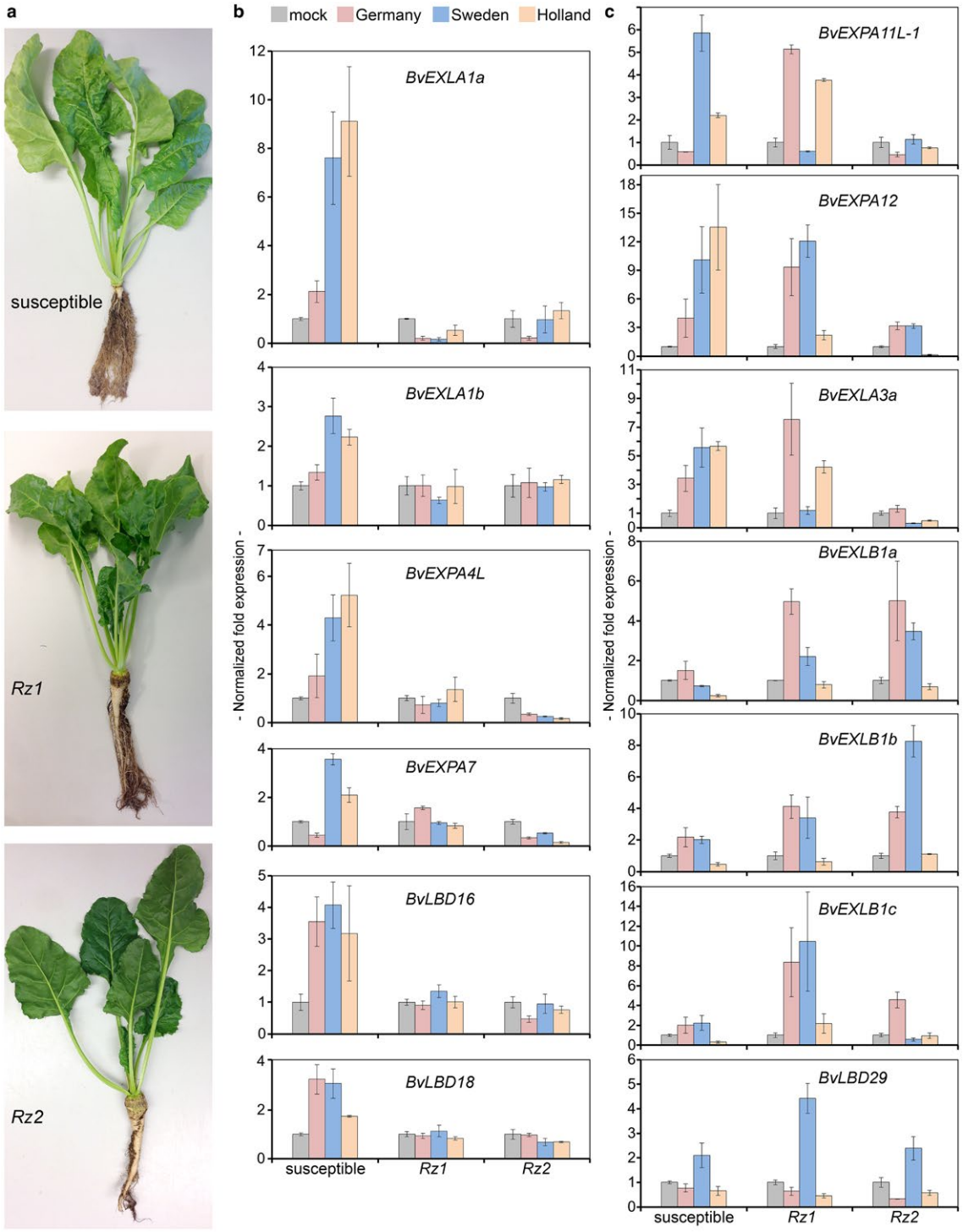


**Fig. 2** Four *EXPANSIN* (*EXP*s) and two *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*s) genes are activated in *Beet necrotic yellow vein virus* (BNYVV)-susceptible, but not BNYVV-resistant, sugar beet varieties, whereas *BvLBD29* and six *EXP*s are up-regulated in both susceptible and resistant varieties, by natural soil-mediated infection with BNYVV. (a) Phenotypes of three different varieties (BNYVV-susceptible, as well as varieties harbouring *Rz1* or *Rz2* resistance genes) grown in soil infested with BNYVV for 6 weeks. (b) Expression of *BvEXLA1a*, *BvEXLA1b*, *BvEXPA4L*, *BvEXPA7*, *BvLBD16* and *BvLBD18* in sugar beet BNYVV-susceptible vs. BNYVV-resistant varieties harbouring *Rz1* or *Rz2* resistance genes at 6 weeks post sowing (wps). (c) Expression of six *EXP*s and *BvLBD29* induced by BNYVV in both susceptible and resistant varieties at 6 wps. Bars represent means ± standard deviation ($n \geq 4$). [Colour figure can be viewed at wileyonlinelibrary.com]

present in the soil, and thus may not directly contribute to rhizomania development. To resolve this issue, we took advantage of the availability of two sugar beet varieties carrying the resistance genes *Rz1* or *Rz2*. Although resistant varieties are infected with BNYVV, they do not develop the disease (Fig. 2a). Therefore, we reasoned that the genes which are induced in susceptible, but not resistant, varieties could be directly linked to rhizomania development.

To compare the induction of *LBD*s and *EXP*s in susceptible vs. resistant varieties, RT-qPCRs were conducted using total RNAs from roots sampled at 6 wps. *BvLBD16*, *BvLBD18*, *BvEXP4L*, *BvEXPA7*, *BvEXLA1a* and *BvEXLA1b* were significantly (Student's two-tailed *t*-test, $P < 0.05$) up-regulated in susceptible (Fig. 2b), but not in resistant, varieties (Student's two-tailed *t*-test, $P > 0.05$; Fig. 2b), suggesting that the activation of these genes contributes to rhizomania development. There was not much change detected in the expression of *BvEXPA7L*, *BvEXPA13* and *BvEXLA2a* (Fig. S3, see Supporting Information). On the other hand, six *EXP*s and *BvLBD29* were activated in both susceptible and resistant varieties, although to a different extent depending on the type of infested soil (Fig. 2c), suggesting that the expression of these genes does not directly contribute to rhizomania

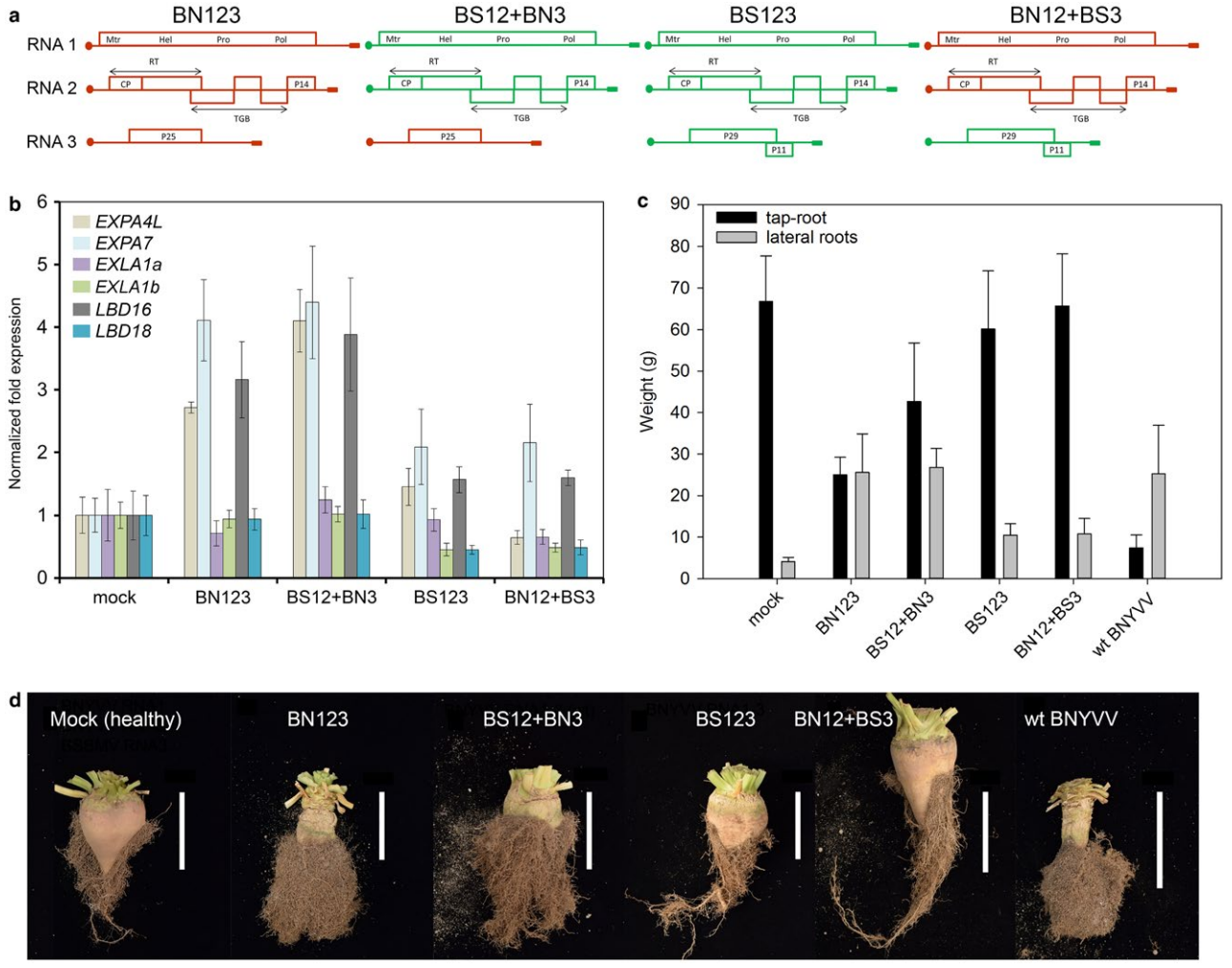


**Fig. 3** Differences in the abundance of *EXPANSIN* (*EXP*s) and *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*s) gene transcripts, and rhizomania development, in *Beta vulgaris* roots on infection with *Beet necrotic yellow vein virus* (BNYVV), *Beet soil-borne mosaic virus* (BSBMV) and interspecies RNA3 reassortants. (a) Schematic representation of the inocula, consisting of BNYVV and BSBMV RNA1, RNA2 and RNA3 genomic segments (BN123 and BS123, respectively) and reassortants for the RNA3 segment (BS12+BN3 and BN12+BS3, respectively), used in the virus infection experiments. (b) Expression of *EXPs and LBD*s in roots of *B. vulgaris* infected with BNYVV (BN123), BSBMV (BS123) and reassortants for the RNA3 segment (BS12+BN3 and BN12+BS3, respectively) at 32 days post-inoculation (dpi). Bars represent means ± standard deviation ($n \geq 6$). (c) Fresh weight of *B. vulgaris* lateral roots (LRs) infected with BNYVV (BN123), BSBMV (BS123) and reassortants for the RNA3 segment at 111 dpi. (d) Symptom appearance in roots of *B. vulgaris* infected with BNYVV (BN123), BSBMV (BS123) and reassortants for the RNA3 segment. Images were taken at 111 dpi. Bars, 5 cm. [Colour figure can be viewed at wileyonlinelibrary.com]

symptoms, but might synergistically enhance the severity of the disease.

To determine whether the presence of BNYVV RNA3, which encodes the P25 virulence factor, is required for the activation of *EXP* and *LBD* genes (identified through the use of *Rz1* and *Rz2* resistant varieties as being important for rhizomania development), we performed infection experiments with recombinant viruses reassorted for RNA3 genomic segments. To this end, we took advantage of the availability of the infectious cDNA clones for BNYVV and *Beet soil-borne mosaic virus* (BSBMV) (Laufer *et al.*, 2018). As for BNYVV, BSBMV belongs to the genus *Benyvirus*, has a genome organization similar to BNYVV and infects sugar beet, but does not cause the 'hairy root' phenotype typical of rhizomania (Workneh *et al.*, 2003). Notably, the BSBMV RNA3-encoded orthologue of P25, the P29 protein, has a very low similarity to P25, namely 23% identity (Lee *et al.*, 2001). Moreover, genomic components can be exchanged between the viruses, as they can be trans-replicated (Ratti *et al.*, 2009). To inoculate the roots of sugar beet seedlings, four different inocula were assembled, each comprising RNA1 + RNA2 of either BNYVV or BSBMV, and RNA3 genomic components reassorted between the two viruses (Fig. 3a). The RT-qPCR analysis performed on total RNA isolated at 32 days post-inoculation (dpi) clearly showed that *BvLBD16*, *BvEXPA4L* and *BvEXPA7* were up-regulated by the presence of BNYVV RNA3, but not by the presence of BSBMV RNA3 (Fig. 3b). Interestingly, we did not observe changes in the level of *BvLBD18*, *BvEXLA1a* and *BvEXLA1b* transcript accumulation, suggesting that, compared with the experiments involving *Rz1* and *Rz2* resistant varieties, the expression of these genes was not induced at this time point.

In another experiment, we evaluated the symptom appearance and fresh weight of LRs infected with reassortant viruses following a growth period that allows the formation of taproot and rhizomania symptoms (111 dpi). Consistent with the RT-qPCR data, the occurrence of symptoms and the increase in fresh weight of LRs correlated with the presence of BNYVV RNA3 in the inoculum (Figs 3c,d and S1c).

### *LBD*s and *EXP*s can be induced by auxin treatment

As the expression of *LBD16/18/29* TFs and some *EXP*s in *Arabidopsis* is controlled through auxin signalling, we asked whether their orthologues in sugar beet could be induced by auxin. To address this question, roots of 3-week-old *B. vulgaris* seedlings were treated with auxin (indole-3-acetic acid, 3-IAA) at low non-toxic concentrations of 10 μM for 2 h. The RT-qPCRs of RNA samples isolated from the roots challenged with 3-IAA revealed significant up-regulation of *BvLBD16*, *BvLBD18* and *BvLBD29*, with *BvLBD29* being up-regulated up to 16-fold relative to mock-treated samples (Fig. 4). Moreover, two of ten *EXP*s tested in these experiments—*BvEXLA1a* and *BvEXPA4L*—were also slightly up-regulated—up to 2.5-fold (Student's one-tailed *t*-test, $P < 0.05$). Thus, these experiments linked auxin signalling to the induction of *LBD*s and *EXP*s in sugar beet and prompted us to analyse auxin signalling components acting upstream of LBD16/18/29.

### *B. vulgaris* AUX-ARF7/19-responsive module genes are not transcriptionally activated by rhizomania development

To determine whether the genes acting upstream of *LBD*s and *EXP*s, but downstream of auxin signalling, are activated by natural soil-mediated infection with BNYVV, total RNA isolated at 6 wps was subjected to RT-qPCR analysis. RT-qPCR results clearly demonstrated that the mRNA levels of *BvIAA14*, *BvARF7* and *BvARF19* either did not show a significant alteration in expression or were slightly down-regulated in comparison with control mock-inoculated plants (Fig. S4, see Supporting Information). These findings are consistent with the notion that the mode of regulation of the AUX-ARF7/19 module occurs through protein–protein interactions and protein stability/turnover.

### P25 interacts with BvAUX28 and the interaction involves BvAUX28 domains I and II

The finding that several *EXP*s and *LBD*s belonging to the LR and RH developmental pathways (in *Arabidopsis*) are up-regulated by rhizomania, and, moreover, that a similar set of genes is induced by auxin treatment of sugar beet roots, raises the possibility

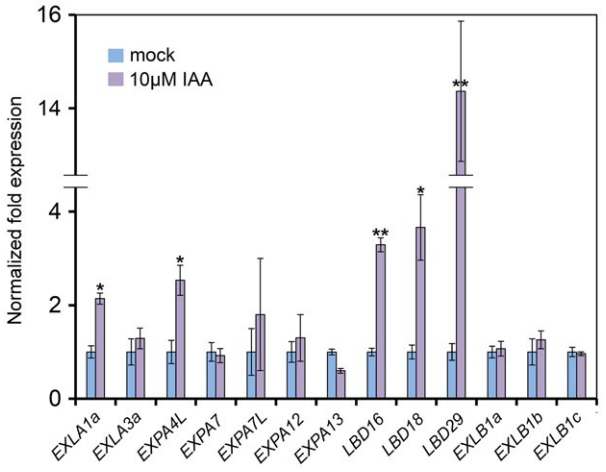


**Fig. 4** Auxin modulates the expression of *EXPANSIN* (*EXP*s) and *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*s) genes in the roots of *Beta vulgaris*. Roots of *B. vulgaris* seedlings were treated with auxin and samples were collected 2 h after treatment with indole-3-acetic acid (3-IAA) or 10% ethanol (mock). Bars represent means ± standard deviation ($n = 3$). $^{*}P < 0.05$, Student's one-tailed *t*-test; $^{**}P < 0.05$, Student's two-tailed *t*-test. [Colour figure can be viewed at wileyonlinelibrary.com]

that *LBD*s and *EXP*s may be induced downstream of auxin signalling via the AUX-ARF7/19-responsive module. Interestingly, a previous study, employing yeast two-hybrid (Y2H) screens, identified a root-specific transcriptional repressor BvAUX28 as

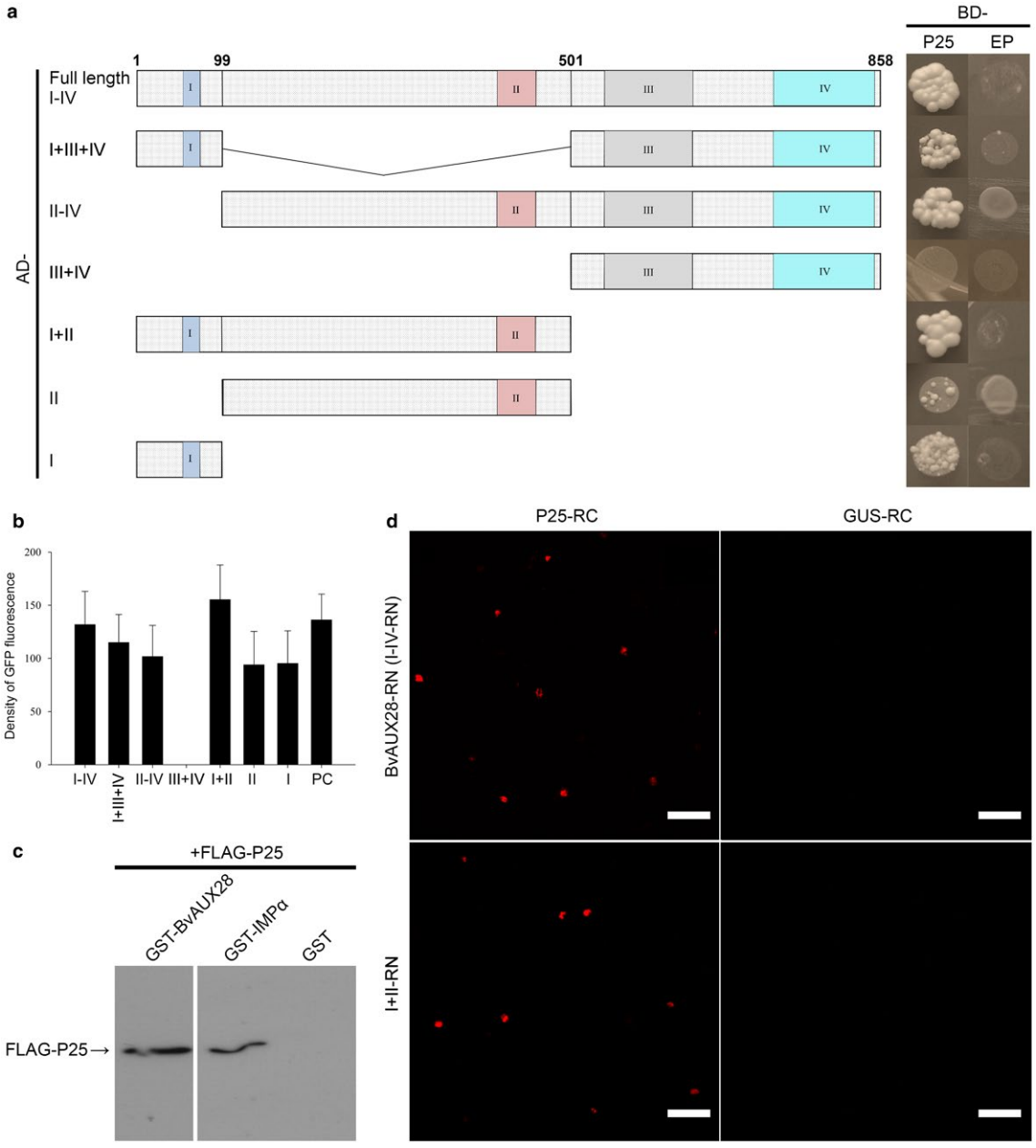


**Fig. 5** The *Beet necrotic yellow vein virus* (BNYVV) P25 protein interacts with BvAUX28. (a) Evaluation of P25 interaction with BvAUX28 in yeast and identification of the interacting domain of BvAUX28. Left: schematic diagram showing the BvAUX28 domains; conserved domains are represented by coloured rectangles. Right: yeast transformants were spotted onto a high-stringency synthetic dropout (SD/-Trp/-Leu/-His/-Ade) medium. Empty BD vector/plasmid (EP) co-transformed with corresponding AD constructs tested for transcriptional autoactivation (negative control). AD, activation domain. BD, binding domain. (b) Relative quantification of leucine-driven green fluorescent protein (GFP) (mean total area density) in diploid yeast expressing the combination of P25 and BvAUX28 or its truncated derivatives as shown in (a). Bars represent means ± standard error of the mean. (c) *In vitro* pull-down assay to confirm P25 interaction with BvAUX28. FLAG epitope-tagged P25 was incubated with glutathione-*S*-transferase (GST)-tagged BvAUX28, GST-tagged OsIMP$\alpha$ (importin $\alpha$ from rice) or with GST tag. GST pull-down products were visualized by immunoblotting with anti-P25 antiserum. (d) Bimolecular fluorescence complementation (BiFC) conformation of the BNYVV P25 and BvAUX28 interaction. P25-RC and BvAUX28-RN (or I+II-RN) were co-expressed in *Nicotiana benthamiana* leaf epidermal cells via agroinfiltration. Co-expression of GUS-RC and BvAUX28-RN or I+II-RN was used as negative control. Images were taken at 4 days post-inoculation (dpi). Scale bars, 50 $\mu$m. [Colour figure can be viewed at wileyonlinelibrary.com]

an interacting partner of P25 (Thiel and Varrelmann, 2009), although the biological role of this interaction has not been addressed. To confirm the interactions between BvAUX28 and P25, we carried out three independent assays. We cloned *BvAUX28* full-length cDNA into a prey plasmid and validated its interactions with P25 in yeast (Fig. 5a,b).

To identify which domain of BvAUX28 is required for the interaction with P25, six constructs expressing various sets of the domains were tested by Y2H assays. The interactions were detected with constructs expressing domains I, II, I+II, II–IV, I+III+IV, as well as full-length BvAUX28 (domains I–IV), whereas a derivative expressing domains III+IV did not show interaction (Fig. 5a). The strength of the P25–BvAUX28 interaction was evaluated by an indirect method involving the quantification of the leucine-driven expression of plasmid-encoded green fluorescent protein (GFP) in yeast (Fig. 5b). When expressed on their own, domains I and II, as well as the II–IV construct, showed weak interaction activity relative to the construct containing both domains I and II (Fig. 5b). Together, these results indicate that the interaction between BvAUX28 and P25 is contributed by domains I and II.

To verify the BvAUX28–P25 interaction, we expressed a recombinant FLAG-tagged P25 protein from a Baculovirus vector in Sf9 insect cells and glutathione-*S*-transferase (GST)-tagged BvAUX28 or GST (as a negative control) proteins in *Escherichia coli*, and performed *in vitro* pull-down assays. As shown in Fig. 5c, FLAG-P25 was detected in GST-BvAUX28, but not in GST pull-down complexes.

To further verify the BvAUX28–P25 interaction *in vivo*, we performed bimolecular fluorescence complementation (BiFC) assay. First, we examined the subcellular localization of BvAUX28 by transiently co-expressing it as a GFP-tagged fusion together with the DsRed-tagged NLS of Simian virus 40 (SV40) T-antigen in *Nicotiana benthamiana* using *Agrobacterium tumefaciens*. As expected, this experiment showed that GFP-BvAUX28 localized exclusively to the nucleus (Fig. S5, see Supporting Information). Next, we verified the interaction between BvAUX28 and P25 *in planta* using BiFC. P25-RC and BvAUX28-RN (or I+II-RN) were transiently co-expressed in *N. benthamiana* leaf epidermal cells and the positive signals of red fluorescent protein (RFP) were detected in the nucleus (Fig. 5d). We did not find any RFP signal in the nucleus when we co-expressed the BvAUX28-RN or I+II-RN

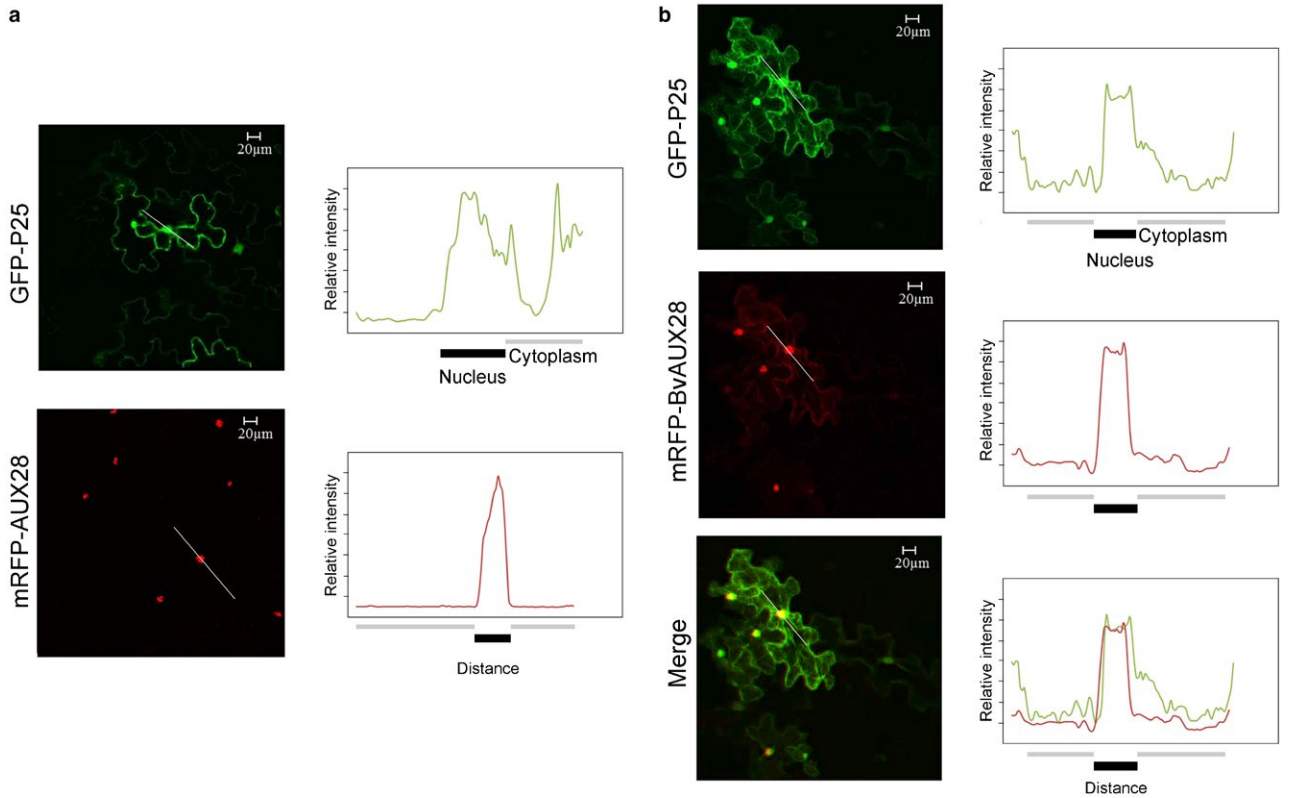


**Fig. 6** *Beet necrotic yellow vein virus* (BNYVV) P25 inhibits BvAUX28 nuclear localization. (a) Intracellular localization of the BNYVV P25 protein and BvAUX28. BNYVV P25-GFP and mRFP-BvAUX28 were transiently expressed in *Nicotiana benthamiana*. (b) Co-expression of BNYVV P25 and BvAUX28. (a, b) Densitometry scans were performed along the lines indicated in white. Different colours indicate P25 (green) and BvAUX28 (red) intensities. Bars below the densitometry scans indicate nucleus (black) and cytoplasm including plasma membrane (grey). GFP, green fluorescent protein; RFP, red fluorescent protein. Scale bars, 20 $\mu$m. [Colour figure can be viewed at wileyonlinelibrary.com]

fusions together with GUS-RC (Fig. 5d). Taken together, these experiments demonstrate that P25 physically interacts with BvAUX28 *in vitro* and *in vivo*.

### P25 inhibits BvAUX28 nuclear localization

We then investigated whether P25 affects the nuclear accumulation of BvAUX28. As expected, the GFP-fused P25 localized to the nucleus and cytoplasm (Fig. 6a). Consistent with its role in the regulation of transcription, the RFP-fused BvAUX28 was exclusively localized to the nucleus in 100% of examined cells (Fig. 6a). To examine the effect of the P25–BvAUX28 interaction on the subcellular localization of BvAUX28, GFP-P25 and RFP-BvAUX28 were transiently co-expressed in *N. benthamiana*. Surprisingly, RFP-BvAUX28 fluorescence, besides localizing in the nucleus, accumulated in the cytoplasm (Fig. 6b). In general, RFP-BvAUX28 fluorescence co-localized with the green fluorescence of GFP-P25 by being detected in the nucleus and cytoplasm (Fig. 6b). Together, these results demonstrate that P25 disrupts the function of BvAUX28 in nuclear accumulation.

## DISCUSSION

BNYVV continues to pose a significant threat to sugar beet production, as exemplified by the emergence of *Rz1* resistance-breaking isolates in different areas of the world (Bornemann *et al*., 2015; Koenig *et al*., 2008; Liu and Lewellen, 2007; Liu *et al*., 2005; Pferdmenges *et al*., 2009). Therefore, there is a growing need to understand the molecular basis of rhizomania disease and to identify the pathways of the host involved in order to develop sustainable solutions for disease management. Although progress is being made in understanding the molecular details of plant–virus interactions in different pathosystems, our knowledge about how viruses interfere with plant development and growth, culminating in diseases, is still insufficient to provide genetic resources for resistance-based breeding in various crops and to develop sustainable methods to control the viruses. Recent advances in the genome sequencing of sugar beet, combined with progress made in understanding the mechanisms and genetic basis of LR emergence and RH induction in the model plant *Arabidopsis*, prompted us to analyse similar pathways operating in the sugar beet crop and their potential contribution to the development of rhizomania disease.

Previous studies examining the transcriptome changes in response to BNYVV in sugar beet (Schmidlin *et al*., 2008) or transgenic expression of P25 in Arabidopsis (Peltier *et al*., 2011) have identified several differentially expressed *EXP* genes, including *AtEXPA14* and *AtEXPA17*, as well as *AtEXPA7* and *AtEXPA18*, which were later shown to be involved in LR development and RH elongation in *Arabidopsis*, respectively (Lee *et al*., 2015). However, it remained unclear which of the functional equivalents of *Arabidopsis EXP*s are up-regulated in sugar beet plants, because different EXPs may play similar roles and converge on similar pathways in various plant species. Therefore, we sought to characterize the *EXP* gene family in *B. vulgaris*, as well as changes in *EXP* expression during rhizomania development. These analyses highlighted at least two virus-reprogrammed pathways, operating in parallel and leading to the development of typical symptoms of rhizomania, namely excessive formation of LRs and RHs.

Among the factors studied during rhizomania development, *EXP*s are primarily known for their role in plant CW remodelling by breaking the hydrogen bonds between cellohexaose and pectin; they thus participate in various aspects of plant development involving cell division and expansion (Cosgrove, 2015), and as potential pro-viral factors (Chen *et al*., 2018; Park *et al*., 2017). The data accumulated from studies in various plant pathosystems, including nematodes, fungi and viruses, suggest that the down-regulation of *EXP*s may represent a defence response (Marowa *et al*., 2016). In *Arabidopsis*, the down-regulation or knockdown of *AtEXLA2* enhances resistance to the necrotrophic fungus *Alternaria brassicicola* (Abuqamar *et al*., 2013). The knockdown of *NbEXPA1* in *N. benthamiana* induces resistance to *Turnip mosaic virus* (TuMV) (Park *et al*., 2017). Furthermore, it has been shown that *NbEXPA1* is a plasmodesmata-specific protein recruited by TuMV to the virus replication complex through its interaction with the viral RNA-dependent RNA polymerase, the NIb protein, and transient expression of *NbEXPA1* promotes TuMV replication and cell-to-cell movement (Park *et al*., 2017). Similarly, the knockdown of *NbEXPA4* expression reduces *Tobacco mosaic virus* (TMV) accumulation in systemically infected plants, whereas the overexpression of *NbEXPA4* accelerates virus replication (Chen *et al*., 2018). The pro-viral role of EXPs is further supported (this study) by the up-regulation of 13 *EXP* genes by natural soil-mediated infection with BNYVV, among which four root-specific *EXP*s are up-regulated in susceptible, but not resistant, varieties, linking the activation of these *EXP*s to rhizomania development.

By integrating RT-qPCR data with the results of Y2H screening into a broader picture, we were able to obtain a model for rhizomania development in sugar beet, which revealed a prominent role of LBD TFs and EXPs in shaping the abnormal growth of the sugar beet taproot. By comparing the expression signatures of *LBD*s and *EXP*s in susceptible and resistant varieties of sugar beet, as well as in *B. vulgaris* roots infected with virus reassortants, for a genomic component encoding the virulence factor of the virus, we identified two *LBD* TFs and four *EXP*s as putative downstream targets, the ectopic expression of which contributes to rhizomania development. Indeed, there was a strong correlation between *BvLBD16*, *BvLBD18*, *BvEXPA7*, *BvEXPA4L*, *BvEXLA1a* and *BvEXLA1b* expression in susceptible varieties and the development of typical rhizomania symptoms. Furthermore, the pro-rhizomania *LBD*s and *EXP*s were either unchanged or down-regulated in *Rz1* and *Rz2* resistant varieties. These findings provide new insights into the

pathways that lead to rhizomania. For example, the activation of *BvEXPA7* and *BvEXPA4L*, both of which have RHE signature motives in their promoters (data not shown) and seem to be involved in RH initiation and elongation, on the one hand, and the up-regulation of *BvLBD16*, *BvLBD18*, *BvEXLA1a* and *BvEXLA1b*, presumably involved in LR development, on the other, suggests that two pathways operate in parallel: one leading to LR emergence and the other leading to RH induction and elongation. Although the question of the increase in root hair number on rhizomania development has not yet been addressed properly, our data support this hypothesis. It also remains to be determined whether there is a cross-talk between these pathways. Intriguingly, some of these genes are induced by the auxin treatment of sugar beet roots, indicating that both pathways are regulated by auxin signalling. Indeed, it has been shown that LR emergence and RH development are auxin-dependent developmental processes (Lee *et al.*, 2015).

Previous studies have reported that plant viruses can hijack the auxin signalling pathway to promote virus infection and virus movement in the host plants (Collum *et al.*, 2016; Jin *et al.*, 2016). The capsid protein of *Rice dwarf virus* (RDV) interferes with auxin signalling by binding to the rice AUX/IAA protein OsIAA10, thereby protecting it from degradation by the 26S proteasome and resulting in typical RDV symptoms in infected plants (Jin *et al.*, 2016). The symptoms include dwarfism, excessive tillering and stunted crown roots, and are phenocopied in transgenic rice overexpressing OsIAA10 or its degradation-resistant derivative (Jin *et al.*, 2016). The TMV replicase physically interacts with three vascular-expressed AUX/IAA proteins, including IAA26 (Padmanabhan *et al.*, 2006). These transcriptional repressors have been suggested to regulate genes involved in phloem loading. TMV infection inhibits the nuclear localization of AUX/IAA proteins, resulting in the transcriptional reprogramming of mature vascular tissue, which is correlated with enhanced TMV spread in the phloem of older leaves (Collum *et al.*, 2016). Taken together, these data are consistent with our findings showing that BNYVV P25 interacts with BvAUX28, presumably inactivating its function as a transcriptional repressor through the disruption of its nuclear localization. Moreover, hormonal content assays measuring levels of auxin in BNYVV-infected sugar beet plants, as well as in *Arabidopsis* plants transgenic for *P25*, have demonstrated significantly elevated levels of auxin relative to those in healthy plants, suggesting a positive feedback loop, presumably leading to further acceleration of LR/RH formation (Peltier *et al.*, 2011; Pollini *et al.*, 1990).

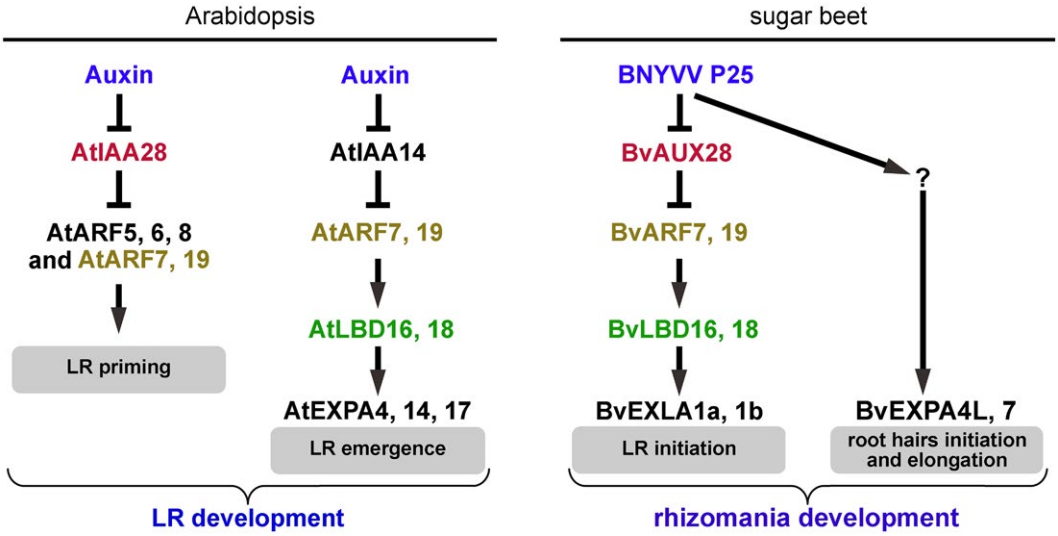


**Fig. 7** Proposed regulatory model for rhizomania development. In healthy *Arabidopsis* plants, auxin controls lateral root (LR) development through multiple auxin signalling modules. Two modules involved in LR development are shown in the left panel (modified from Lavenus *et al.*, 2013 and Lee *et al.*, 2015). LR founder cell priming involves the AtIAA28-AtARF5, 6, 7, 8, 19 module. LR emergence is controlled by the AtIAA14-AtARF7, 19 module. AtLBD16, 18 regulate LR emergence downstream of AtARF7, 19. In sugar beet, following *Beet necrotic yellow vein virus* (BNYVV) infection, the P25 virulence factor is produced. P25 inactivates BvAUX28 transcriptional repressor leading to the derepression of BvARF7, 19 transcriptional activators. Subsequently, the expression of a number of their target genes is turned on, including BvLBD16 and BvLBD18 transcription factors (TFs) involved in LR formation. BvLBD16, 18 activate the expression of two EXLA1 genes to facilitate cell wall (CW) relaxation and remodelling during LR initiation and emergence. The up-regulation of the positive regulators of LR formation, BvLBD16 and BvLBD18, as well as EXPANSIN genes (*EXP*s), leads to the uncontrolled formation of LR, giving a 'hairy root' appearance of rhizomania-afflicted sugar beet taproot. Meanwhile, another pathway is turned on leading to the activation of root hair (RH)-specific *EXPs, BvEXPA4L* and *BvEXPA7*, to promote RH initiation and elongation. The formation of LRs and RHs is beneficial for the propagation of *Polymyxa betae*, the formation of zoospores and, ultimately, the efficient transmission of BNYVV. Auxin and P25 are shown in blue. The proteins encoded by homologous genes in *Arabidopsis* and sugar beet are shown by similar colours. [Colour figure can be viewed at wileyonlinelibrary.com]

Therefore, here, we propose a regulatory model for rhizomania development integrating our present data in sugar beet and previous reports on LR and RH development in *Arabidopsis* (Fig. 7). In healthy plants, LR and RH formation is tightly regulated. Auxin activates the signalling pathways, inducing LR founder cell priming, through the degradation of root-specific AUX/IAA transcriptional repressors (Fig. 7; left panel). Another auxin-dependent pathway also controls RH formation. In BNYVV-infected plants, the P25 virulence factor physically interacts with BvAUX28 and disrupts its function in nuclear accumulation, perhaps as a result of nuclear to cytoplasmic shuttling of P25, thus preventing BvAUX28 interaction with ARFs. Hence, ARFs are derepressed and activate the expression of *LBD*s, promoting further LR development. In turn, LBDs activate the expression of *EXP*s for CW loosening and remodelling, facilitating cell separation for LR formation. Another pathway must be operating in parallel and leads to the up-regulation of *BvEXPA4L* and *BvEXPA7*, presumably needed for RH formation and elongation.

We argue that the formation of LRs and RHs is beneficial for *P. betae*, the vector of BNYVV. The life cycle of *P. betae* and other plasmodiophorids starts with a primary zoospore that attaches to the wall of an RH often in the zone of elongation (Ahm and Buchenauer, 1999; Barr and Asher, 1996). Moreover, during the vegetation period, sporosori of plasmodiophorids could be observed in RHs of the infected plants, suggesting that RHs are the main sites of plasmodiophorid propagation. Hence, *P. betae* propagation and the formation of sporosori are beneficial for BNYVV transmission to increase the number of zoospores and the probability of infecting further neighbouring plants.

To conclude, our study further extends the range of plant developmental processes affected by viruses through interference with phytohormone signalling. Our work identifies at least two pathways for the development of rhizomania and paves the way for follow-up studies, e.g. on the detailed characterization of the molecular mechanism of AUX28 inactivation by P25 and its contribution to rhizomania development. It remains to be determined whether nuclear export or import of AUX28 is affected by P25, and whether interaction of AUX28 with P25 leads to AUX28 degradation via the 26S proteasome. Although these aspects of P25 functioning are fascinating, they are beyond the scope of this article and will be addressed in future studies.

## EXPERIMENTAL PROCEDURES

### Virus isolates and plant inoculation

The sources of BNYVV isolates used in this study were soil samples from Thurnhof, Germany (B-type BNYVV), Landskrona, Sweden (B-type) and Holland (A-type). The B-type isolates have been partially sequence characterized (Koenig and Lennefors, 2000; Lennefors *et al.*, 2006). Sugar beet inbred pollinator lines from the MariboHilleshog breeding pool (susceptible, line 13012502; *Rz1*, line 15014536; *Rz2*, line 14109005) were germinated in sterile sand and 1-week-old seedlings were transplanted into 0.25-L plastic tubes, which contained BNYVV-infested soil mixed with sterile sand (1 : 10 ratio). Each treatment included at least ten plants. The experiment was repeated twice.

### Plant material and growth conditions

Tubes with sugar beet plants were cultivated in a quarantine glasshouse under controlled conditions (day, 22 °C/16 h; night, 20 °C/8 h). *Beta macrocarpa*, *B. vulgaris* and *N. benthamiana* plants were cultivated under glasshouse conditions (day, 24 °C/14 h; night, 18 °C/10 h).

### Sequence alignments and phylogenetic analysis

Full-length protein sequences of *B. vulgaris* and *A. thaliana* EXPs were retrieved from GenBank (Benson *et al.*, 2013). Using MEGA version 7 (Kumar *et al.*, 2016), multiple alignments were made with the algorithm MUSCLE with default parameters (Edgar, 2004), followed by phylogenetic reconstruction by neighbour joining. The topology of the tree was tested using bootstrap (1000 replications) and a Poisson model was used as substitution model.

### RT-qPCR and detection of BNYVV and *P. betae* by RT-PCR

Total RNA from leaves was extracted with a MagJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Total RNA (1 μg) was transcribed into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). Four microlitres of one-tenth-diluted cDNA were used for qPCR with the DyNAmo Flash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). This reaction was supplemented with 0.125 μM of each primer. The *BvICDH* (isocitrate dehydrogenase) gene was used as reference. The resulting qPCR data were analysed using the 2-ΔΔCt method. The sequences of the primers used for qRT-PCR are provided in Table S5 (see Supporting Information).

For pathogen detection, BNYVV- and *P. betae*-specific primers were used (Table S5). The reaction was set with initial denaturation of 95 °C for 1 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 5 min.

### Plant inoculation with recombinant viruses

Reassortment experiments in *B. vulgaris* were conducted using infectious full-length cDNA clones of BNYVV and BSBMV (Laufer *et al.*, 2018). Different combinations of viral RNAs were first inoculated into *B. macrocarpa* leaves using agroinfiltration with *A. tumefaciens*. Then, leaves of *B. macrocarpa* displaying symptoms of systemic infection were used for vortex

inoculation of 7-day-old *B. vulgaris* seedlings, as reported previously (Bornemann and Varrelmann, 2011). BNYVV- and BSBMV-specific enzyme-linked immunosorbent assay (ELISA) of infected LRs was performed to determine the virus content, as described previously (Pferdmenges *et al.*, 2009).

### Auxin treatment of sugar beet seedlings

Auxin treatment was carried out as follows: 21-day-old sugar beet seedlings (KWS03) were treated with 10 μM 3-IAA (Sigma-Aldrich, St. Louis, Missouri, USA) applying vacuum infiltration for 2 min (González-Lamothe *et al.*, 2012); seedlings were kept in infiltration solution for a further 2 h in the dark at room temperature; subsequently, RNA was extracted and subjected to RT-qPCR analysis.

### Y2H assays

The Grow'n'Glow Y2H system (MoBiTec, Göttingen, Germany) used is a modified version of the LexA (pEG202 as bait) and B42 (as prey) yeast two-hybrid system (Gyuris *et al.*, 1993). In addition, it contains a LexA operator-controlled GFP reporter plasmid pGNG1 (Cormack, 1998). The analysis of the optical density of the total area of GFP fluorescence was carried out by epifluorescence microscopy of yeast cells using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The yeast vector pJG4-5 (MoBiTec) carrying a partial cDNA clone of BvAUX28 with domains I–II was named pJG4-5-I-II, and was obtained from a P25 Y2H screen of a sugar beet cDNA library (Thiel and Varrelmann, 2009). The complete BvAUX28 open reading frame (ORF) was cloned from sugar beet breeding line MS150 (Syngenta, Landskrona, Sweden). To obtain constructs carrying full-length as well as truncated derivatives of the BvAUX28 ORF, PCR products were cloned into *EcoR*I-*Xho*I sites giving rise to plasmids named pJG4-5-I, pJG4-5-II, pJG4-5-II-IV, pJG4-5-III-IV and pJG4-5-I+III-IV. The truncated derivative constructs contain BvAUX28 sequences encoding the conserved domains I (amino acids 1–99), II (amino acids 100–501), II–IV (amino acids 100–858), III–IV (amino acids 502–858) and I + III–IV (amino acids 1–99 + amino acids 502–858).

### GST pull-down assay

FLAG epitope-tagged P25 was produced in Sf9 insect cells infected with recombinant baculovirus. The *E. coli* BL21-codon Plus (DE3) RIL strain (Stratagene, La Jolla, California, USA) was used to express the GST fusion proteins. The *in vitro* GST pull-down experiments were performed as described previously (Vetter *et al.*, 2004). The expression of the protein from pGEX-BvAUX28 was performed at 28 °C. Aliquots of the supernatants were analysed using 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were immunodetected using anti-P25 immunoglobulin G (IgG).

### Transient expression and live cell imaging of fluorescent proteins

For subcellular localization and BiFC studies, all constructs were delivered by agroinfiltration to *N. benthamiana* leaves. *Agrobacterium tumefaciens* (strain C58C1/pGV2260) cells were electro-transformed with the plasmid constructs. *Agrobacterium tumefaciens* cultures carrying the plasmids were grown overnight (16 h) at 28 °C. Bacterial cells were pelleted by centrifugation at 1200 × g for 10 min, and then resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM 2-(N-morpholino)ethanesulfonic acid, 150 μM acetosyringone) prior to infiltration. Confocal imaging of GFP- and mRFP-expressing leaf tissues was performed with a Leica, Wetzlar, Germany TCS SP5 confocal imaging system applying excitation and emission wavelengths of 488 and 510–515 nm for GFP, and 514–561 and 600–630 nm for mRFP.

### BiFC assay

A BiFC system with an optimized mRFP was chosen for protein–protein analysis in *N. benthamiana* epidermal leaf cells (Zilian and Maiss, 2011). Therefore, P25, *BvAUX28* and *BvAUX28-I+II* were amplified with specific oligonucleotides (Table S5) and cloned into the vectors pES264 and pES265 using *Bam*HI or *Bgl*II and *Sal*I. The plasmids obtained were named pES264-P25, pES265-I-IV and pES265-I-II. The coat protein (CP) of *Plum pox virus* (PPV) displaying self-interaction served as a positive control, whereas β-glucuronidase (GUS) was used as a negative control.

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## AUTHOR CONTRIBUTIONS

EIS and MV conceived the research plans; JFG, SL, B-LL, TK, DG, EM, MV and EIS designed the research; EIS, JFG, SL and HT performed the experiments; JFG, SL, B-LL, TK, DG, MV and EIS analysed the data; EIS wrote the paper with contributions from all authors.

## CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Fig. S1** Detection of *Beet necrotic yellow vein virus* (BNYVV) and *Polymyxa betae*, and comparison of P25 sequences from BNYVV strains used in our experiments.

**Fig. S2** Thirteen *EXPANSIN* (*EXP*) and three *LATERAL ORGAN BOUNDARIES DOMAIN* (*LBD*) genes are up-regulated by natural soil-mediated infection with *Beet necrotic yellow vein virus* (BNYVV).

**Fig. S3** *BvEXPA7L*, *BvEXPA13* and *BvEXLA2a* are not transcriptionally activated by natural soil-mediated infection with *Beet necrotic yellow vein virus* (BNYVV) in both susceptible and resistant varieties.

**Fig. S4** *BvIAA14*, *BvARF7* and *BvARF19* are not transcriptionally activated by natural soil-mediated infection with *Beet necrotic yellow vein virus* (BNYVV).

**Fig. S5** BvAUX28 localizes to the nucleus.

**Table S1** The *EXPANSIN* genes in sugar beet (*Beta vulgaris*).

**Table S2** Relative expression of B*eta vulgaris EXPANSIN*s in different organs measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

**Table S3** Beet necrotic yellow vein virus (BNYVV)-dependent changes (by natural soil-mediated infection with BNYVV) in EXPANSIN gene expression at 4 and 6 weeks post-sowing (wps) assayed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

**Table S4** Beta vulgaris orthologues of Arabidopsis genes involved in lateral root development used in our analysis.

**Table S5** Sequences of all oligonucleotides used in this study.