The α-Helical D1 Domain of the Tobacco bZIP Transcription Factor BZI-1 Interacts with the Ankyrin-repeat Protein ANK1 and Is Important for BZI-1 Function, Both in Auxin Signaling and Pathogen Response*

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The tobacco (Nicotiana tabacum) bZIP transcription factor BZI-1 is involved in auxin-mediated growth responses and in establishing pathogen defenses. Transgenic plants expressing a dominant-negative BZI-1-ΔN derivative, which lacks the N-terminal activation domain, showed altered vegetative growth. In particular auxin-induced rooting and formation of tobacco mosaic virus-induced hypersensitive response lesions are affected. BZI-1-related proteins described in various plant species share the conserved domains D1, D2, BD, and D4. To define those BZI-1 domains involved in transcription factor function, BZI-1 deletion derivatives were expressed in transgenic plants. The domains D1 or BD are crucial for BZI-1-ΔN function in planta. The basic BD domain is mediating DNA binding of BZI-1. Yeast two-hybrid and in vitro binding studies reveal the ankyrin-repeat protein ANK1, which specifically interacts with a part of the BZI-1 protein (amino acids 73–222) encoding the D1 domain. ANK1 does not bind DNA or act as a co-activator of BZI-1-mediated transcription. Moreover, green fluorescence protein localization studies propose that ANK1 is acting mainly inside the cytosol. Transcription analysis reveals that ANK1 is ubiquitously expressed, but after pathogen attack transcription is transiently down-regulated. Along these lines, ANK1 homologous proteins in Arabidopsis thaliana have been reported to function in pathogen defense. We therefore propose that the D1 domain serves as an interaction surface for ANK1, which appears to regulate BZI-1 function in auxin signaling as well as pathogen response.

The tobacco bZIP protein BZI-1 displays all the characteristic features of a transcription factor. It binds DNA, in particular ACGT containing cis-elements (ACEs), it is localized inside the nucleus, and its N-terminal domain acts as a trans-activation domain in plant cells (1). Like CPRF2, a highly homologous bZIP protein from parsley, BZI-1 has been isolated by virtue of its in vitro binding to chalcone synthase promoter cis-elements (1–4). However, using various transgenic approaches that modulate the amount or the activation potential of BZI-1, we were not able to show any influence on transcription of phenylpropanoid pathway genes, such as chalcone synthase or phenylalanine ammonia lyase in vivo (1).

Functional analysis has been performed in transgenic plants expressing a dominant-negative BZI-1 derivative lacking the N-terminal activation domain (BZI-1-ΔN). These plants display reduced internodes, small curly leaves, enhanced lateral shoot formation, and flowers that are reduced in size. In particular auxin responses appeared to be reduced with respect to auxin-induced rooting and regulation of a GH3 target gene. Moreover, BZI-1 transcription is up-regulated in response to pathogen attack and pathogen-induced phosphorylation of BZI-1-related proteins has been described (1, 3, 4).

BZI-1-related transcription factors have been isolated from various plant species, e.g. CPRF2, (2), OHP1/2 (5), BLZ1 (6), or bZIP63 (7). Apart from the N-terminal activation domain, BZI-1-related transcription factors share several highly conserved domains (1). Since transcription factors show a modular architecture, it seems likely that these conserved domains mediate specific functions. The D1 domain displays a α-helical structure, frequently localized in protein–protein interaction surfaces. The D2 domain harbors many acidic residues, as it has been described for activation domains (8).

Analyzing the parsley bZIP factor CPRF2, a phytochrome-mediated nuclear import was described. Making use of a CPRF2-GFP fusion, sequences related to the D1 and D2 domains have been postulated to act as cytosolic retention domains (9). The basic domain (BD) of other bZIP factors was shown to mediate DNA binding and nuclear localization (10). The ZIP domain of BZI-1 facilitates specific homo- or heterodimerization (3). Finally, a phosphorylation site has been mapped in the C terminus of the BZI-1-related transcription factor CPRF2 (11).

In this work, transgenic plants expressing deletion derivatives of BZI-1 have been used to define protein domains essential for BZI-1 function in planta. The basic BD domain establishes DNA binding and is involved in nuclear translocation of BZI-1. The D1 domain appears to be crucial for BZI-1 function in the context of auxin signaling or pathogen defense response. Frequently, protein–protein interactions play an important role in signaling processes (12). To isolate proteins specifically interacting with the α-helical D1 domain, a yeast two-hybrid screen has been performed. The interaction partner, an ankyrin-repeat protein referred to as ANK1, is unable to bind...
DNA or to modulate transcription as a co-factor. Moreover, the GFP-ANK1 fusion protein is localized inside the cytosol. ANK1 transcription is transiently down-regulated after pathogen attack, and TMV-induced HR formation is affected in BZI-1-ΔN plants. We therefore propose that protein interaction between ANK1 and BZI-1, mediated by the D1 domain, is involved in auxin and/or pathogen defense signaling.

EXPERIMENTAL PROCEDURES

Plant Cultivation and Pathogen Infection—Tobacco plants (Nicotiana tabacum cv. Xanthi NN) were grown in a growth chamber under a 16-h light/8-h dark cycle at 22 °C and 85% humidity. For TMV infection, fully expanded leaves of 6- to 8-week-old tobacco plants, which were grown in soil, were inoculated with TMV strain U1 (1 μg per leaf) in 50 mM potassium phosphate buffer, pH 7.0, or “mock” inoculated with buffer by gently rubbing the leaves with carborundum using a method which has been described previously (15, 16). Organogenesis was carried out by cultivating tobacco explants on solid Murashige and Skoog medium (17) supplemented with 0.2 mg liter⁻¹ naphthalene acetic acid and 1 mg liter⁻¹ 6-BAP.

Standard Molecular Biological Techniques—Standard DNA techniques have been described in Ref. 18. DNA sequence analysis was performed using an ABI310 sequencer, utilizing an ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit.

Vector Construction—A HA-tagged BZI-1 fusion gene (HA-BZI-1-ΔN) was obtained by inserting synthetic oligonucleotides (pHA1, CCGGGT-ACAATGCGTGACCTTTCAAGCTGACCTGACCCACCG; pHA2, CCGTCCGCTTCAAGCTGACCTGACCCACCG; pHA3, CCTGACCCATCCGACCTGACCCACCG; pHA4, CCTGACCCATCCGACCTGACCCACCG) into the KpnI and BamHI restriction sites at the 5' end of the BZI-1 coding sequence. The plasmid obtained was named pUCATT-x-HA-BZI-1-ΔN. The deletion BZI-1-ΔNAD2 was generated by digesting pUCATx-HA-BZI-1-ΔN with XbaI and BamHI. Insertion of the oligonucleotides O135 (CTGCAAGCTTCAACCATCTG) and O136 (GATCCATCCCTGAGCTT) rebuilt the BZI-1 reading frame (BZI-1-ΔNAD2). The BZI-1-ΔNAD1 derivative was obtained from the plasmid pET28-BZI-1-ΔN (3). Deletion of an internal Dral fragment followed by religation resulted in pET28-BZI-1-ΔNAD1. The BZI-1-ΔNAD1 and BZI-1-ΔNAD2 fusion vectors have been used to construct yeast and plant transformation vectors.

To express a plasmid for in vitro transcription/translation, pGEM-ANK1 was constructed. The ANK1 gene was PCR-amplified using the primer mK2 (CCATGGACCTGCAGAACAACACATCTTTCTC) and mk3 (GGAAGAAGAAGACGACGTGGCCACACATCTGGACCCA). The PCR fragment was cloned into vector pGEM-T (Promega, Madison, WI).

Plant Transformation—The plant transformation vectors pBIN-BZI-1-Oex and pBIN-BZI-1-ΔN were constructed. pBIN-HA-BZI-1-Oex was derived from the plasmid pHBT-BZI-1 or pHBT-BZI-1-ΔN. The interaction between BD-p53 and AD-SV40 large T-antigen was used in vitro -galactosidase assays were performed using a confocal microscope (LSM-510, Zeiss). GFP localization studies have been carried out according to Ref. 1). 1. Northern blot method was performed using a BZI-1-specific XhoI/PstI restriction fragment (1).

RESULTS

The Protein Domains N, BD, and D1 Are Important for BZI-1 Transcription Factor Function in Plants—Conserved protein domains have been defined in BZI-1-related proteins by means of amino acid (aa) homology (1). To analyze a putative role of these domains in BZI-1 function, BZI-1 deletion derivatives were expressed in transgenic plants (Fig. 1, A and B). Whereas BZI-1-overexpressing tobacco plants (BZI-1-Oex) do not display any visible alterations, expression of a dominant-negative BZI-1-ΔN derivative, which lacks the N-terminal activation domain, resulted in plants showing a complex phenotype characterized by reduced internodes, increased development of lateral shoots, and size-reduced curvy leaves (Fig. 1B). Therefore, the N-terminal domain is crucial for BZI-1 function in planta (1).

Phenotypic alterations imply that these plants might be affected in hormone signaling. Using leaf explants derived from BZI-1-ΔN plant in an organogenesis assay, cytokinin-induced rooting was unchanged (data not shown), but auxin-induced rooting appeared to be significantly reduced (Fig. 1E). Further, BZI-1 has been implicated to function in the context of pathogen defense (1, 4). Hence, the transgenic plants were infected with TMV (Fig. 1F). Whereas viral spread was limited in wild type plants as monitored by reference to localized HR lesion formation, BZI-1-ΔN plants showed a spreading of the HR lesions, which resulted in a breakdown of the whole leaf tissue.

These phenotypic alterations have been used to map BZI-1 protein domains. BZI-1-ΔN derivatives were expressed in transgenic tobacco, displaying additional deletions of the conserved domains. In all assay systems described above, deletion of the D2 domain did not alter the BZI-1-ΔN phenotype, implying that this domain might be not important for BZI-1-ΔN function. In contrast, deletion of D1 resulted in wild type plants, abolishing the function of the overexpressed BZI-1-ΔN protein.

a 1. Lenk, unpublished data.
Deletion of protein domains might alter expression, protein stability, nuclear localization, or DNA binding properties of BZI-1. We therefore performed a number of controls to molecularly characterize the transgenic plants. Expression of the constructs was examined by Northern (Fig. 1C) and Western analysis (Fig. 1D). All transgenic plants used for further studies showed significant protein levels of the BZI-1 derivatives. Using recombinant proteins, BZI-1-H9004N and BZI-1-H9004N/H9004D1 were found to bind ACEs in EMSA (Fig. 2). Hence, these factors still can function as DNA-binding proteins. In contrast, the deletion of the DNA binding domain in BZI-1-H9004N/H9004BD abolishes DNA binding (Fig. 2), implying that this protein is inactive in controlling specific promoter targets. Using transiently transformed protoplasts, BZI-1-GFP fusion protein was detected predominantly in the nucleus (1). Like BZI-1-GFP, the BZI-1-H9004N-GFP, BZI-1-H9004N/H9004D1-GFP, or BZI-1-H9004N/H9004D2-GFP fusion proteins were found in the nucleus. Compared with BZI-1-GFP, the nuclear localization of these proteins appeared to be slightly enhanced (Fig. 3, d–f), whereas BZI-1-H9004N/H9004BD-GFP was enriched in the cytosol (Fig. 3g). Nevertheless, complete nuclear exclusion,
as it was found for a cytosolic protein (RanBP1a-GFP) (19), could not be observed for BZI-1/H9004 N/BZI-1/H9004 BD-GFP.

Like BZI-1/H9004 N, BZI-1/H9004 N/H9004 D1 protein is stable in planta, binds DNA, and is localized in the nucleus. However, since BZI-1/H9004 N/H9004 D1 plants appeared like wild type, the presence of the D1 domain seems important for establishing the BZI-1/H9004 N phenotype.

The BD domain is important for DNA-binding and is involved in nuclear localization. However, its deletion does not completely abolish function of the overexpressed BZI-1 derivative. Although the vegetative part of BZI-1/H9004 NBD transgenic plants appears like wild type (Fig. 1B), it has to be noted that slight alterations, e.g. in flower size (data not shown), occur. Auxin-induced rooting shows an intermediate phenotype that varies during repetitions of assay. However, TMV infection results in wild type lesion formation (data not shown). In conclusion, the influence of BZI-1NBD on BZI-1 related transcription appears to be complex.

Isolation of the Ankyrin-repeat Protein ANK1 That Interacts with the D1 Domain of BZI-1—Since the D1 domain shows an α-helical structure frequently found in protein interaction domains, a yeast two-hybrid screen was performed to identify BZI-1 interaction partners. As a bait, an internal fragment encoding the domains D1 and D2 (aa 73–244) was fused with the GAL4 DNA binding domain (GALBD) (3) (Fig. 4A). Expression of the bait construct in yeast was monitored by means of Western analysis (Fig. 4B). Screening of 10⁷ clones resulted in the isolation of a cDNA encoding a 37-kDa protein, referred to as N. tabacum ANK1 (Fig. 5A). In silico analysis revealed that the C-terminal part of ANK1 harbors four ankyrin repeats, protein domains typically involved in protein-protein interaction (for a review see Ref. 25). Compared with the ankyrin repeat consensus sequence, the repeats 2 and 3 were well conserved, whereas repeats 1 and 4 displayed some alterations. An almost identical gene sequence has been isolated from N. tabacum cv. SNN (HBP1, GenBank™ accession number AAL25088), but functional data have not yet been published. The homologous proteins AKR2 (MIPS At4g35450) (13) and AtPhos43 (MIPS At2g17390) (14) have been described in Arabidopsis thaliana (Fig. 5A). Apart from the N-terminal 50 aa, which are less conserved, these proteins share a high aa identity of 66%. The N-terminal 50 aa are rich in proline, aspartic acid, serine, and threonine, as has been described for the PEST domains (23).
ANK1 has been found reproducibly in the yeast two-hybrid screen (19 independent clones). The interactions have been monitored by adenine prototrophic growth of the yeast strains (data not shown) and quantitative β-galactosidase enzyme activity assay (ONPG-Assay) (Fig. 4C). To map the ANK1 interaction domain, BZI derivatives were constructed, deleting parts of the domains D1 or D2, respectively (for details see Fig. 4A). Since the N-terminal 73 aa lead to auto-activation, the deletions were constructed based on the BZI-1 N terminus bait. However, the deletion in D1 only results in a 50% decrease in ANK1-BZI-1 interaction. Hence, D1 is involved in the interaction with BZI-1, but the deleted aa 101–152 are not sufficient. Therefore, we assume that the neighboring aa participate in ANK1 binding. In contrast, deleting the central part of D2 shows that this domain is not participating in ANK1-BZI-1 interactions. Furthermore, the C-terminal part of the BZI-1 protein did not show any interaction with ANK1. In summary, we conclude that the region between aa 73 and 222 acts as an interaction surface, which is sufficient for BZI-1-ANK1 interaction.

ANK1 Does Not Bind DNA, but Depending on the D1 Domain, ANK1 Inhibits DNA Binding of BZI-1 in Vitro—In

**Fig. 4. Isolation of N. tabacum ANK1 by means of a yeast two-hybrid screen.** A, schematic drawing of the BZI-1-derived bait (HA-BZI-1-(73–244)) used in a yeast two-hybrid screen. The corresponding part of the BZI-1 aa sequence (1) showing the conserved domains D1 and D2 (boxed sequence) is illustrated. The aa deleted in the bait proteins BD-HA-BZI-1-D1ΔN or BD-HA-BZI-1-D2ΔN are indicated. To verify expression in yeast, a HA epitope tag (black bar) was included in the bait construct located in between BD and BZI-1. BD, GAL4 DNA binding domain. B, Western blot showing the BD-HA-BZI-1-(73–244) bait protein detected by means of a HA epitope-specific antibody. Yeast cells harboring the plasmids pGBT9 and pGBT-BZI-1-(73–244) are analyzed, respectively. C, ONPG assay measuring BZI-1 ANK1 interaction by means of a lacZ reporter. Co-transformants carrying BZI-1-derived bait vectors and a pGAD-ANK1 prey vector were analyzed. As controls, autoactivation of pGBT-derivatives was measured. The mean values of three independent measurements are given, and the experiment was repeated three times. As a positive control, the interaction between BD-p53 and AD-SV40 large T-antigen was used (Clontech MATCHMAKER system).
EMSA, BZI-1-ΔN homodimers were found to bind ACEs, but ANK1 does not bind ACE-related DNA sequences (Fig. 6). Interaction between BZI-1 and ANK1 in yeast was confirmed in the *in vitro* assay. ANK1 inhibits binding of recombinant BZI-1-ΔN protein, whereas BZI-1-ΔNΔD1 protein co-incubated with ANK1 binds DNA. As has been found in yeast, deletion of the D1 domain does not completely abolish BZI-1-ANK1 interaction. In agreement with the *in vitro* data obtained for yeast and *in planta*, *in vitro* protein interaction shows that the α-helical surface (aa 101–152) deleted in BZI-1-ΔNΔD1 participates in ANK1 interaction but is itself not sufficient.

**ANK1 Does Not Act as a Co-factor of BZI-1-mediated Transcription**—Since ANK1 interacts with a transcription factor it might regulate transcription indirectly by acting as a co-factor. In yeast, expression of a GALBD fusion with ANK1 leads to significant activation of the GAL-UAS-lacZ reporter (Fig. 7A). Hence, by interacting with BZI-1, ANK1 might function as a co-factor modulating BZI-1-mediated transcriptional control. Expression of a GALBD-BZI-1 protein in tobacco protoplasts resulted in a significant activation of a 4xGAL-UAS-GUS reporter (Fig. 7B). The level of activation was comparable to that of the strong activator GALBD-VP16. However, co-expression of GALBD-BZI-1 and ANK1 did not enhance transcription of the reporter, as it can be assumed from a co-activator. In contrast, a slight reduction of transcription has been observed, which is not significant. To verify that ANK1 expression has no indirect effects on cell viability, co-transfection with a functional unrelated GALBD-VP16 protein has been performed. In this control, ANK1 expression did not interfere with normal cell activities.

**The ANK1 Protein Is Mainly Localized in the Cytosol**—In silico analysis of BZI-1 did not show any well defined nuclear localization sequences. An ANK1-GFP fusion gene was transiently expressed in tobacco protoplasts, and cellular localization was analyzed by confocal microscopy. Whereas non-fused GFP was found in the nucleus and in the cytosol (Fig. 3a), ANK1-GFP was mainly localized in the cytosolic compartment (Fig. 8). As a control, exclusion outside the nucleus can be seen for RanBP1a-GFP fusion protein as has been described previously (19) (Fig. 3b). However, this clear exclusion of the nucleus cannot be seen with ANK1-GFP protein. Hence, ANK1 is mainly localized in the cytosol, but we cannot rule out that small amounts might be present in the nucleus.

According to the model proposed in Ref. 9, nuclear translocation of the BZI-1-related transcription factor CPRF2 is regulated by a cytosolic retention protein. If ANK1 were to act as a cytosolic-retention factor, co-expression should result in alterations in BZI-1 localization. However, after co-expression of ANK1, we could not detect any significant changes in cellular localization of the BZI-1-ΔN-GFP fusion protein (data not shown).

**ANK1 Transcription Is Transiently Repressed after Pathogen Attack**—ANK1 has been found to be constitutively expressed in all plant tissues tested (Fig. 9A). Since BZI-1 is involved in mediating auxin and pathogen defense responses, we tested whether ANK1 expression is regulated by these stimuli. Infection with *Pseudomonas syringae* (Fig. 9B) results in a transient decrease in the ANK1 transcript level. Comparable results were obtained with other elicitors, e.g. cryptogein (data not shown). The efficiency of infection was controlled by activation *EAS4*, a pathogen-inducible gene (24). No effect on ANK1 transcription was observed after treatment with auxin in response to light/dark rhythms or wounding (data not shown). Hence, ANK1 transcription specifically responds to the pathogen stimulus.

**DISCUSSION**

In this work we have mapped protein domains conserved among BZI-1-related bZIP transcription factors with respect to their function in auxin and pathogen signaling. The BD domain has been shown to be involved in DNA binding. The α-helical D1 domain was found to be important for BZI-1 function in *planta*. Most likely D1 acts as a protein interaction surface for...
ANK1, a protein characterized by ankyrin repeats.

The α-Helical Domain D1 Is Crucial for BZI-1 Transcription Factor Function—In general, transcription factors are characterized by a modular structure. Functions such as DNA binding, transactivation, nuclear localization, multimerization, or interaction with regulatory partners are mediated by distinct protein domains. Ongoing exchange and combination of these modules provides a mechanism to establish new functions within transcription factor families. Several conserved protein domains have been described in BZI-1-related proteins (1, 4), implying they might be essential for transcription factor function. Overexpression of BZI-1-N, which lacks the N-terminal activation domain, affect auxin and pathogen signaling. The BZI-1-N protein has been shown to bind DNA and to be localized to the nucleus as it has been described for the full-size BZI-1 protein. The bZIP domain of BZI-1 has been shown to be a specific heterodimerization domain, interacting with the tobacco bZIP factors BZI-2, BZI-3, and BZI-4, respectively (3). We therefore propose that BZI-1-N acts as a dominant-negative factor, binding promoter target sites as non-activating homo- or heterodimers. Furthermore, by means of binding with the ankyrin repeat protein ANK1, overexpressed BZI-1-N protein might titrate ANK1 protein, which in turn cannot function in interaction with endogenous BZI-1 transcription factor. BZI-1-NBD also should titrate the ANK1-interacting factor; nevertheless the phenotype is more related to wild type. These data argue against the titration hypothesis. However, we cannot rule out that the deletion of the BD domain affects interaction with ANK1 in planta. In summary, BZI-1-N appears to affect cellular functions by various mechanisms.

Expression of deletion derivatives of the BZI-1-N protein in transgenic plants provides a valuable tool to identify those domains essential for BZI-1-N function. Although BZI-1-NBD protein has been shown to be stable in transgenic plants, the protein is impaired in DNA binding as confirmed by EMSA analysis. As it has been demonstrated for other bZIP transcription factors, such as OPAQUE2 (10), the basic domain contains a bipartite nuclear localization signal. However, in the protoplast system used, BZI-1-NBD concentration appears...
to be slightly increased in the cytosol; however, it is not excluded from the nucleus. The residual nuclear import of BZI-1-D1ΔNΔBD may result from an additional nuclear localization sequences, miss-localization of overexpressed GFP fusion proteins, or co-transport with other heterodimerizing bZIPs. In some aspects, the BZI-1-D1ΔNΔBD plants showed an altered phenotype. Since DNA binding, interaction with ANK1, nuclear import, and most likely heterodimerization is affected, the BZI-1-D1ΔNΔBD protein might interfere with endogenous BZI-1 function in a more complex manner.

Because of the high amount of acidic aa, the D2 domain has been suggested to act in transcriptional activation. However, D2 was not involved in transcriptional activation in yeast. Furthermore, BZI-1-D1ΔD2 plants showed the BZI-1-DN phenotype, implying that the D2 domain is not important for establishing the BZI-1-DN phenotype in planta. However, we cannot completely rule out a function of D2 involved in wild type BZI-1 function.

Although the BZI-1-D1ΔD1 protein was stable in transgenic plants, can bind DNA, and is localized in the nucleus, its expression does not result in the BZI-1-DN phenotype. Hence, the D1 domain appears to play a crucial role in BZI-1 signaling. In consequence, BZI-1-DN does not act by simply blocking promoter target sites; in addition to DNA binding properties, the D1 domain is necessary for transcription factor function.

In Arabidopsis a genome-wide classification of bZIP transcription factors led to the identification of the subgroup C, which harbors BZI-1-related proteins (7). Group C transcription factors, as well as BZI-1-related bZIPs from other monocot and dicot plants, harbor D1-related sequences. In contrast, D1 domains are not encoded in other bZIP subgroups such as group S, which includes specific BZI-1 interaction partners. We therefore propose that the domain is important for the function of group C-related bZIP transcription factors.

ANK1 Defines a Specific Interaction Partner of the BZI-1 Domain D1—Using a yeast two-hybrid screen, ANK1 has been isolated as a BZI-1 interaction partner. This interaction is of functional relevance because (i) ANK1 has been isolated repeatedly in the screen and (ii) the interaction is specific to a defined part of the BZI-1 protein. Since no interaction with the closely situated D2 domain was observed, we can define the ANK1 interaction domain as being from aa 73 to 222. (iii) The BZI-1 D1 domain shows an α-helical structure, which has been described as mediating protein-protein interactions. (iv) The interaction was verified in an in vitro system, and (v) the D1 interaction domain was found to be of functional relevance in planta when deleted in a BZI-1 derivative.

The ANK1 protein contains four ankyrin repeats of 33 conserved aa. The ankyrin motif consists of two α-helices, surrounded by two β-sheets on both sides. Consequently, the ankyrin repeat forms a surface for protein-protein interaction (25). The number of ankyrin-repeats varies between different proteins. Ankyrin-repeat proteins are involved in various functional aspects; for example, mammalian IκB is involved in nuclear retention of the NFκB transcription factor (26) or NPR1 in Arabidopsis interacts with members of the TGA class of bZIP transcription factors and in turn regulates the transcription of PR1 (27, 28). No functional conclusion can be drawn for proteins harboring this domain. Nevertheless, ankyrin-repeat containing proteins share typical features of signaling components that have the potential to form specific protein interactions (12).

ANK1-BZI-1 Protein-Protein Interaction, Implications for Auxin and Pathogen Signaling—After TMV infection, initiation of HR lesions is unchanged in BZI-1-DN plants, but a restriction of the lesion size is impaired. This kind of cell death shows a marked resemblance to the propagation-class mutant phenotype described, for example, in Arabidopsis lsd1 mutants (29). LSD1 was found to be a zinc finger protein, which acts as a negative regulator of cell death (29, 30). Accordingly, after triggering cell death, BZI-1-DN plants probably lack this negative feedback regulation, limiting execution of the cell death program (31, 32).

The ANK1 homologous genes from Arabidopsis thaliana are involved in plant pathogen defense reactions. In particular Yan et al. (13) have isolated AKR2 by using a yeast two-hybrid screen. AKR2 interacts with a 14-3-3 protein (GF14γ) and ascorbate peroxidase, which scavenges reactive oxygen species (ROS) as an antioxidant. AKR2 antiseptate plants result in the induced formation of ROS and form spontaneous lesions. ROS are known to be essential signaling molecules involved in initiation and propagation of HR lesions (31), and they are postulated to participate in auxin-regulated developmental processes (33, 34). In Arabidopsis, the dth9 mutant shows increased susceptibility to P. syringae infection as well as auxin insensitivity (35, 36). It is tempting to speculate, that ROS are involved in cross-talk between auxin and pathogen defense signaling pathways and that protein interactions between BZI-1 and ANK1-like proteins are essential in this context.

As it has been described for the NFκB/IκB system in mammals (26), Yan et al. (13) suggested that AKR2 might act as a cytosolic retention protein that regulates nuclear trans-localization of a presently unidentified transcription factor (13). Like IκB, ANK1 or AKR2 harbor ankyrin repeats and a PEST domain. PEST domains are involved in protein degradation. After stimulation, rapid phosphorylation of the PEST domain results in IκB ubiquitination and thereby targets this protein for degradation at the proteasome (37). Consequently, the NFκB transcription factor is no longer retarded in the cytosol and can regulate its target genes inside the nucleus. The ANK1 homologue AtPhoe43 has been isolated by using a proteomic approach, as a protein which is rapidly phosphorylated in response to flagellin elicitor treatment (14). The question whether this phosphorylation triggers degradation of the protein has not yet been studied. We were able to show that tobacco ANK1 is transcriptionally down-regulated after elicitation. Protein degradation, as well as transcriptional repression, would result in a transient decrease in ANK1 protein and might complement each other. Using ANK1-GFP fusion proteins, we were able to show that ANK1 is mainly localized in the cytosol. Hence, ANK1 would fulfill the requirements necessary for being a retention factor regulating nuclear translocation of BZI-1. However, NFκB homologous proteins have not been detected in Arabidopsis genome projects (38). In this study, we were able to demonstrate that ANK1 specifically interacts with BZI-1, a bZIP transcription factor involved in auxin signaling and pathogen defense. Moreover, nuclear uptake of CPRF2, the putative BZI-1 orthologue isolated from parsley has been found to be regulated depending on sequences corresponding to the D1 and D2 domains (9). A putative cytosolic retention factor has been postulated, which regulates this nuclear import. These data would fit in nicely with the proposed model. However, using BZI-1-GFP fusion proteins we were not able to confirm that ANK1 prevents nuclear translocation of BZI-1-GFP or that elicitors enhance nuclear uptake of BZI-1-GFP. Nevertheless, since highly overexpressed GFP fusion proteins were used in transfected protoplasts, this experimental set-up might not be close to the natural situation. In planta immunolocalization studies might be more suited to reveal ANK1 function. According to this model, ANK1 is supposed to be a negative regulator of BZI-1 transcription factor function. After pathogen attack, ANK1 is inactivated and BZI-1
moves into the nucleus to regulate yet undescribed genes involved in cell death.

In an alternative model, ANK1 is proposed to be a positive regulator of BZI-1 function. Hence, BZI-1 target genes are involved in an unknown mechanism that prevents cell death. After pathogen attack ANK1 function is transiently reduced, involving an unknown mechanism that prevents cell death. Along these lines, BZI-1ΔN is proposed to titrate ANK1 from interaction with BZI-1 and thereby enhances spreading of HR lesions. Identification of BZI-1 target genes, as well as analysis of BZI-1 and ANK1 RNAi in plants will be valuable tools to further reveal the mechanism of ANK1 function.

No evidence was found indicating that ANK1 is directly involved in transcriptional control but most likely functions by forming protein complexes outside the nucleus, e.g., by regulating storage, nuclear translocation, or modification of BZI-1 transcription factors. Moreover, pathogen-induced transient decrease of the ANK1 protein level provides a stimulus-induced mechanism that regulates the BZI-1 transcription factor.

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