Senescence-associated Barley NAC (NAM, ATAF1,2, CUC) Transcription Factor Interacts with Radical-induced Cell Death 1 through a Disordered Regulatory Domain*\\n
Received for publication, April 4, 2011, and in revised form, August 17, 2011 Published, JBC Papers in Press, August 19, 2011, DOI 10.1074/jbc.M111.247221

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Background: Plant NAC transcription factors (TF) are important regulators of senescence. Senescence-associated barley HvNAC013 uses intrinsic disorder in transcriptional activation and interactions. Radical induced cell death 1 exploits the intrinsic disorder of HvNAC013 and other TFs for interactions without structure induction in HvNAC013. This first structural characterization of NAC intrinsic disorder may reveal general features of important TF regulatory interactions.

Senescence in plants involves massive nutrient relocation and age-related cell death. Characterization of the molecular components, such as transcription factors (TFs), involved in these processes is required to understand senescence. We found that HvNAC005 and HvNAC013 of the plant-specific NAC (NAM, ATAF1,2, CUC) TF family are up-regulated during senescence in barley (Hordeum vulgare). Both HvNAC005 and HvNAC013 bound the conserved NAC DNA target sequence. Computational and biophysical analyses showed that both proteins are intrinsically disordered in their large C-terminal domains, which are transcription regulatory domains (TRDs) in many NAC TFs. Using motif searches and interaction studies in yeast we identified an evolutionarily conserved sequence, the LP motif, in the TRD of HvNAC013. This motif was sufficient for transcriptional activity. In contrast, HvNAC005 did not function as a transcriptional activator suggesting that an involvement of HvNAC013 and HvNAC005 in senescence will be different. HvNAC013 interacted with barley radical-induced cell death 1 (RCD1) via the very C-terminal part of its TRD, outside of the region containing the LP motif. No significant secondary structure was induced in the HvNAC013 TRD upon interaction with RCD1. RCD1 also interacted with regions dominated by intrinsic disorder in TFs of the MYB and basic helix-loop-helix families. We propose that RCD1 is a regulatory protein capable of interacting with many different TFs by exploiting their intrinsic disorder. In addition, we present the first structural characterization of NAC C-terminal domains and relate intrinsic disorder and sequence motifs to activity and protein-protein interactions.

Transcription in eukaryotes is complex and involves a large number of transcription factors (TFs). Gene-specific TFs bind to regulatory regions of target genes and thereby enhance or impede the progression of RNA polymerase. The DNA-binding domain (DBD) is the defining feature of TFs and is used for gene family assignment. TFs also contain a transcription regulatory domain (TRD). TRDs do not have well-defined sequence motifs and structures and are often classified according to regions enriched in specific amino acids, e.g. glutamine.

These characteristics have hampered prediction of protein interacting regions in TRDs.

TRDs are often characterized by intrinsic disorder (ID), referring to an ensemble of protein conformers without a single stable tertiary structure and with fluctuating secondary structures of low population. The function of disordered regions in TFs relates to molecular recognition, which is often, but not always, linked to folding upon binding. The flexibility associated with ID allows TFs to interact efficiently with a number of different target proteins. Binding by regions with ID can be mediated by molecular recognition features (MoRFs), which are short sequence stretches with propensities to undergo disorder to order transition upon binding. Binding regions can also be predicted from linear motifs (LMs), referring to sequence patterns in proteins binding a common target.

Although MoRFs are not associated with a specific sequence motif an overlap often exists between a LM and a MoRF. LMs

* This work was supported by Danish Agency for Science Grant 274-07-
0173 (to K. S. and P. G.) and the Villum Kann Rasmussen Foundation (to M. K. J.).
The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3 and Table S1.

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The abbreviations used are: TF, transcription factor; NAC, NAM, ATAF1,2, and CUC; TRD, transcription regulatory domain; RCD1, radical indicated cell death 1; DBD, DNA-binding domain; ID, intrinsic disorder; MoRF, molecular recognition feature; LM, linear motif; NAM, no apical meristem; CUC, cup-shaped cotyledon; NACBS, NAC DNA-binding site; ERD1, early responsive to dehydration stress 1; NTL6, NTM1 (NAC with transmembrane motif 1)-like 6; NAP, NAC-like, activated by AP3/PI, ore1, oresara1; PONDR, predictor of natural disordered regions; MEME, multiple expectation maximization for motif elicitation; TFE, trifluorethanol; HSQC, heteronuclear single quantum coherence; VND, vascular-related NAC domain; palNACBS, palindromic NAC DNA-binding site; RST, RCD1, SRO, TAF4, SANT, Swi3, Ad2, N-CoR, TFIIB; VNI2, VND-interacting2; Ribisco, ribulose-bisphosphate carboxylase/oxygenase.
NAC Transcription Factor Intrinsic Disorder in Interactions

To improve understanding of the NAC TF-associated regulatory mechanisms of senescence we present extensive insights in structure-function relationships of two senescence-induced NAC genes, HvNAC005 and HvNAC013, from barley. The NAC domain of both proteins bound the NACBS, but only HvNAC013 activated transcription. To understand this at a molecular level we present the first structural characterization of a NAC TRD. The C-terminal domains of both proteins were largely intrinsically disordered. However, only the domain of HvNAC013 interacted with HvRCD1. Other types of TFs also interacted with RCD1 through regions with ID. We discuss the implications of ID in NAC and other TFs for regulatory interactions with RCD1 and different functionality of modularly similar NAC TFs involved in senescence.

EXPERIMENTAL PROCEDURES

Accession Numbers—The following accession codes were used: AtbHLH11, At4g36060; AtMYB91, At2g37630; AtRCD1, At1g32230; HvNAC005, AK251058; HvNAC-013, AK376297.1; and HvRCD1, FR846236.

Bioinformatics Tools—ID was predicted using PONDR VL3 or Disopred2 (26, 27). MEME (multiple EM (expectation maximus) for motif elicitation) was used to identify sequence motifs. Multiple alignments were made by Clustal X (28), and BLAST at the National Center for Biotechnology Information was used to analyze sequences.

cDNA Synthesis and Quantitative Real-time PCR (qRT-PCR)—RNA was isolated from ~100 μg of frozen homogenized material from Golden Promise cultivar using the Spectrum™ Plant Total RNA kit (Sigma) and recommendations. First strand cDNA synthesis was performed as described (25). qRT-PCR was performed using the ABI Prism 7900HT Sequence Detection System with the Power SYBR® Green PCR master mix (Applied Biosystems). HvNAC005, HvNAC013, and HvRCD1 were cloned with primers flanking the coding regions (supplemental Table S1). The cDNA template used in the PCR was synthesized from senescent or green leaf tissue.

Production and Purification of Proteins—Specific primers (supplemental Table S1) were used to amplify NAC fragments with a tobacco etch virus or thrombin protease cleavage site. The PCR products were inserted into pDEST17 (Invitrogen) to obtain glutathione transferase (GST)-tagged protein. His6-NAC005 and His6-NAC013(1–346) were cleaved using tobacco etch virus protease (Invitrogen).

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Electrophoretic Mobility Shift Assay (EMSA)—This was performed as described (14).

Size Exclusion Chromatography—Analytical size exclusion chromatography was performed on a Superdex™ 75 10/300 GL column with an Äkta 900 Purifier (GE Healthcare). Standard proteins for calibration were: myoglobin (17.6 kDa), GST (26 kDa), bovine serum albumin (66 kDa); blue dextran was used to determine the void volume ($V_0$). Proteins were analyzed in 20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM DTT. Stokes radii were according to Ref. 30.

Assays in Yeast—Bait and prey fragments were fused to GAL4 DBD or transactivation domain in pGBK7/pGADT7 (Clontech) (supplemental Table S1). HvNAC013 mutants were constructed using the QuikChange mutagenesis kit (Stratagene), and assayed as described (8). Some plates contained 3-amino-1,2,4-triazole as indicated to inhibit self-activation.

In Vitro Interaction Studies—HvNAC013 and HvRCD1 interaction were analyzed by intrinsic fluorescence spectroscopy using the QuikChange mutagenesis kit (Stratagene), and assayed as described (8). Some plates contained 3-amino-1,2,4-triazole as indicated to inhibit self-activation. β-Galatosidase activity was measured using a liquid culture assay and ortho-nitrophenyl-β-galactosidase substrate. Fragments of AtRCD1, amplified from cDNA from the Arabidopsis Biological Resource Center, and BHLH11 or MYB91, amplified from REGIA TF cDNA, were recombined into pDEST32/pDEST22 (Invitrogen). Protein extracted as described (16) was detected by Western blotting using GAL4-DBD monoclonal antibodies (Clontech) or anti-tubulin antibodies as loading control.

Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR) Spectroscopy—Far-UV CD spectra were recorded on a Jasco 810 spectropolarimeter with a 1-mm path length from 250 to 190 nm, 20 nm/min, 8 accumulations, and 2-s response time. Sample conditions were 5 M protein in 10 mM NaH2PO4/Na2HPO4, pH 7.0. Buffer backgrounds were subtracted. Trifluoroethanol (TFE) concentrations were 0–40% (v/v). The spectra were smoothed using a fast Fourier transform marker genes of senescence, encoding a papain-like cysteine peptidase (Cyspep) and Rubisco small subunit (Rubisco) were used as markers of senescence, known to be up-regulated and down-regulated, respectively (32). Nonsenescent (NS) represents a young green flag leaf at ear emergence, 15 days (15D) a flag leaf 15 days after ear emergence and senescent (Sen) a highly chlorotic, but still fully turgid, flag leaf. Data are represented as mean ± S.E. (error bars) from 3 independent experiments ($n = 3$).

RESULTS

Expression of Barley HvNAC005 and HvNAC013 Is Induced by Senescence—Senescence in plants involves nutrient relocalization and age-related cell death (24, 32), and identification and characterization of molecular components of these processes are required to understand senescence. Recently, barley HvNAC005 and HvNAC013 were identified as genes up-regulated during senescence (25). To investigate their expression pattern in more detail, a time course experiment was performed. Three stages of flag leaf development were investigated, starting with young green leaves and ending with fully senescent, although still turgid, leaves (Fig. 1). We included two marker genes of senescence, encoding a papain-like cysteine peptidase (AM941122) and the Rubisco small subunit (ABK79421), respectively (32). This showed that the expression levels of HvNAC005 and HvNAC013 were up-regulated as senescence progressed. Interestingly, HvNAC005 and HvNAC013 were expressed at staggered intervals. The expression of HvNAC005 was increased more strongly than that of HvNAC013 and showed an 8-fold increase 15 days after ear emergence. HvNAC005 and HvNAC013 were induced ~20- and 13-fold, respectively, in fully senescent leaves compared with nonsenescent leaves.

HvNAC005 and HvNAC013 Belong to Phylogenetically Different Subgroups—To investigate the phylogenetic clustering of HvNAC005 and HvNAC013, an unrooted tree of predicted barley NAC proteins (25) was constructed based on their conserved NAC domains (Fig. 2). This showed that HvNAC005 and HvNAC013 belong to distant subgroups. To further access subgroup specificity, Arabidopsis, wheat, and rice NAC proteins implicated in senescence (21, 22, 24, 33–35) were included in the tree. This together with BLAST searches revealed that HvNAC005 is closely related to NAP from Arabidopsis (21). HvNAC013 belongs to a group, which in addition to its closest Arabidopsis homologue, ANAC046, contains ORE1 and the CUC and vascular-related NAC domain (VND) proteins (8, 36).
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**FIGURE 2.** *HvNAC005* and *HvNAC013* belong to different phylogenetic NAC protein subgroups. Phylogenetic presentation of the barley NAC TF family. The analysis is based on the NAC domain part of the predicted barley NAC TF sequences (25). Alignments and the tree were constructed using ClustalX 2.1 (28). Six NAC genes, known from previous studies to be involved in senescence regulation, were also included (indicated with asterisks); Arabidopsis NAP (21), wheat NAM-B1(24), Arabidopsis ORE1 (22), Arabidopsis NTL9 (33), Arabidopsis ORS1 (35), and rice NAC5 (34).

*HvNAC005* and *HvNAC013* Have Classic DNA-binding NAC Domains and C-terminal Domains with Intrinsic Disorder—The *HvNAC005* and *HvNAC005* cDNAs were obtained from senescent barley leaves and shown to encode proteins of 346 and 355 amino acid residues, respectively (supplemental Fig. S1). The NAC domain of both *HvNAC005* and *HvNAC005* showed a high degree of sequence similarity to NAC domains from typical NAC proteins (supplemental Fig. S1) (8). In a previous study, ANAC019, representing the NAC subgroup of *HvNAC005*, and NAC2/ORE, representing the NAC subgroup of *HvNAC013*, were used as probes to select a NACBS containing CGT[G/A] as core sequence (14). A palindrome version of NACBS (palNACBS), which was used previously to examine NAC DNA binding (8, 14), was used to examine the in vitro DNA-binding properties of *HvNAC005* and *HvNAC013* (Fig. 3A). Purified GST-tagged recombinant versions of the NAC domains, GST-35421/HvNAC005(1–172) and GST-35421/HvNAC013(1–176), were used to examine the abilities of *HvNAC005* and *HvNAC013* to bind palNACBS in titration series using from 10 to 500 ng of protein. Both *HvNAC005* and *HvNAC013* bound palNACBS, in contrast to GST. For *HvNAC013*, binding could be detected using 10 ng of protein (Fig. 3C), whereas *HvNAC005* showed weaker affinity with binding observed using 100 ng of protein (Fig. 3B), which is similar to affinities observed for other NAC proteins (8). The contribution of specific bases and amino acid residues to the interaction between the NACBS and NAC TFs has not been characterized in molecular details. To ensure the importance of the NACBS for binding, EMSAs were, therefore, also performed using an oligonucleotide where the two NACBSs were replaced by simple nucleotide repeats (Fig. 3A) (14). No binding was detected with substituted palNACBS (Fig. 3, B and C). Fig. 3, D and E, shows that binding of both *HvNAC005* and *HvNAC013* to palNACBS was outcompeted by unlabeled palNACBS (lanes 3–5) but not by substituted palNACBS (lanes 6–8).

We previously predicted the structurally divergent C-terminal domains of Arabidopsis NAC proteins to be intrinsically disordered (8). To investigate if *HvNAC005* and *HvNAC013* share this characteristic they were examined for ID using the predictor PONDR VL3 (26). This analysis suggested that *HvNAC005* and *HvNAC013* have a large degree of disorder within their C-terminal domains, although they show different distributions of disorder (Fig. 4). *HvNAC013* displayed an ~100 amino acid residues stretch with ID within the C terminus (Fig. 4B). Conversely, *HvNAC005* showed ID immediately following the NAC domain followed by a stretch of order before another stretch with ID (Fig. 4A).

*HvNAC013* Is a Transcriptional Activator with Several Regions Contributing to Transcriptional Activity—Many NAC C termini are TRDs (8, 37). These domains are diverse and contain subgroup-specific sequence motifs (8). The MEME motif search tool was used to identify motifs shared between *HvNAC013* and its closest homologues from barley and rice (Fig. 2). Four motifs with conservation of both polar and non-polar amino acid residues were identified (Fig. 5, A and B) and named either after motifs reported previously (LP motif) (37) or after prominent amino acid residues characteristic of the motif (DV, YF, RR). Whereas the LP motif is frequent in NAC TRDs (8), the other three motifs are specific for a small subgroup of crop NAC TFs. Similar analyses also revealed sequence motifs in *HvNAC005* (supplemental Fig. S1).

Coding regions of *HvNAC005* and *HvNAC013* were fused to yeast GAL4 DBD (Fig. 5A) and analyzed for their ability to activate transcription and promote yeast growth in the absence of histidine and adenine. Murine tumor suppressor p53 (pVA3-1) and SV40 large antigen (pTBD1-1) were included as positive controls for transformation and activity dependent selective growth (38). Full-length *HvNAC013* was able to activate transcription, and the activation potential resided in the C terminus (residues 177–346), whereas the N-terminal NAC domain (residues 1–176) was unable to activate reporter genes (Fig. 5C). Surprisingly, neither truncated nor full-length *HvNAC005* had transactivation activity in yeast (Fig. 5C).

C-terminal deletions were made to examine the importance for transcriptional activity of the identified motifs (Fig. 5A). The analysis showed that the region containing the LP motif is sufficient for transcriptional activity (Fig. 5C). Mutations were introduced in the LP motif to change the highly conserved leucine and aspartate, which could participate in protein-protein interactions. Examination of *HvNAC013*(L212A) and *HvNAC013*(D217A) showed that both mutant proteins were active in the yeast growth assay (Fig. 5C).

Quantitative β-galactosidase assays in yeast showed a high level of transcriptional activity for full-length *HvNAC013* and for its C terminus, as expected (Fig. 5D). These levels were comparable with that obtained by activation caused by interaction between the positive control p53 and SV40 large antigen (38). A stepwise decrease in activity was detected by C-terminal trun-
cations removing identified sequence motifs, and activity was completely abolished by removal of the LP motif region. Both L212A and D217A mutant versions resulted in a significant decrease in activity compared with wild type HvNAC013 (Fig. 5D). The level of expression of the GAL4 DBD fusion proteins was examined by Western blotting. This showed expression of all proteins with the expected molecular mass values and no obvious correlation between protein expression levels and activity; i.e., the variance in protein level did not explain the lack of activation activity of, e.g., HvNAC005 and HvNAC13(1–200), and the decreased level of activity of HvNAC013(L212A) and HvNAC013(D217A) (Fig. 5E). In conclusion, the TRD of HvNAC013 has a complex structure in which distinct sequence motifs or regions in concert contribute to activity. Only deletion of all motifs abolished activity.

The HvNAC005 and HvNAC013 C Termini Are Intrinsically Disordered but Can Adopt α-Helix Structure—To examine the structure of the C-terminal domains of HvNAC005 and HvNAC013 experimentally, these domains were produced as recombinant proteins with an N-terminal histidine (His6) tag. The proteins, which were produced in E. coli BL21(DE3)pLysS, were purified by metal affinity chromatography using standard procedures (29). Although the His6 tag had no significant influ-

### Table: Predictions of structure and disorder in HvNAC005 and HvNAC013

| Domain       | PalNACBS | PalNACBS, substituted |
|--------------|----------|-----------------------|
| NAC domain   | PalNACBS | PalNACBS, substituted |
| HvNAC005     | PalNACBS | PalNACBS, substituted |
| Disorder     | PalNACBS | PalNACBS, substituted |

### Figure 3: Analysis of HvNAC005 and HvNAC013 NAC domain DNA binding using EMSAs
- A, sequences of the palindromic and substituted NAC binding sequence, palNACBS and palNACBS substituted, used for EMSA. Bases in the core NAC binding motif are indicated in bold. C, binding of HvNAC005 and HvNAC013 to palNACBS. EMSAs using the oligonucleotides palNACBS (second to fifth lanes) or palNACBS substituted (sixth to ninth lanes) and 0, 10, 100, or 500 ng GST-HvNAC005(1–172) or GST-HvNAC013(1–176). 300 ng of GST (first lane) was used as negative control. D and E, sequence specificity of HvNAC005 and HvNAC013 DNA binding. First lane, no protein. Second to eighth lanes, 100 ng of HvNAC005 or 10 ng of HvNAC013. Binding was competed by the addition of none (second lane), 3 times (third and sixth lanes), 25 times (fourth and seventh lanes), and 125 times (fifth and eighth lanes) molar excess of unlabeled palNACBS or palNACBS substituted. Bound DNA is indicated with arrows.

### Figure 4: Predictions of structure and disorder in HvNAC005 and HvNAC013
- A, PONDR VL3 (26) analysis of HvNAC005 (A) and HvNAC013 (B). A threshold is applied with disorder assigned to values greater than or equal to 0.5 as indicated by the black bar.
NAC Transcription Factor Intrinsic Disorder in Interactions

HvNAC013 is a transcriptional activator with several sequence regions contributing to transcriptional activity. A, schematic diagram showing full-length and deletion mutants of HvNAC013 and HvNAC005 fused to the GAL4 DBD. Amino acid substitutions are indicated by crosses. A black bar indicates NAC domain boundaries. Sequence motifs are indicated by gray bars. B, weblogs of MEME sequence motifs identified in the TRD of HvNAC013 and its close homologs. The relative height of a letter in the WebLogo indicates its relative frequency at the given position (x axis). C, yeast transactivation assay. Fusion proteins of GAL4 DBD and HvNAC013 or HvNAC005 fragments were expressed in yeast and screened for their transactivation activity on the HIS3 and ADE2 reporter genes. Empty pGBK7 served as negative control and pVA3-1 and pTD1-1 as positive controls. D, quantitative β-galactosidase assays. The cells were assayed for β-galactosidase activity using ortho-nitrophenyl-β-galactoside as substrate. Activity was measured in Miller units (x axis). Data are represented as mean ± S.E. (error bars) from 3 independent experiments (n = 3). E, level of expressed GAL4 DBD fusion proteins. Fusion proteins of HvNAC005 and HvNAC013 were expressed in yeast strain pJ694A and detected by Western blot analysis with GAL4-DBD monoclonal antibodies or anti-tubulin antibodies as loading control.

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FIGURE 5. HvNAC013 is a transcriptional activator with several sequence regions contributing to transcriptional activity. A, schematic diagram showing full-length and deletion mutants of HvNAC013 and HvNAC005 fused to the GAL4 DBD. Amino acid substitutions are indicated by crosses. A black bar indicates NAC domain boundaries. Sequence motifs are indicated by gray bars. B, weblogs of MEME sequence motifs identified in the TRD of HvNAC013 and its close homologs. The relative height of a letter in the WebLogo indicates its relative frequency at the given position (x axis). C, yeast transactivation assay. Fusion proteins of GAL4 DBD and HvNAC013 or HvNAC005 fragments were expressed in yeast and screened for their transactivation activity on the HIS3 and ADE2 reporter genes. Empty pGBK7 served as negative control and pVA3-1 and pTD1-1 as positive controls. D, quantitative β-galactosidase assays. The cells were assayed for β-galactosidase activity using ortho-nitrophenyl-β-galactoside as substrate. Activity was measured in Miller units (x axis). Data are represented as mean ± S.E. (error bars) from 3 independent experiments (n = 3). E, level of expressed GAL4 DBD fusion proteins. Fusion proteins of HvNAC005 and HvNAC013 were expressed in yeast strain pJ694A and detected by Western blot analysis with GAL4-DBD monoclonal antibodies or anti-tubulin antibodies as loading control. Lanes 1, pGBK7 vector control; 2, HvNAC005(173–355); 3, HvNAC005(1–220); 4, HvNAC005(1–250); 5, HvNAC013(1–200); 6, HvNAC013(1–355); 7, HvNAC013(1–346)(D217A); 8, HvNAC013(1–346); 9, HvNAC013(1–175); 10, HvNAC013(1–220); 11, HvNAC013(1–346); 12, HvNAC013(1–346)(L211A); 13, pVA3-1; pTD1-1.
showed a clustering of signals with low dispersion in the proton dimension (Fig. 6D) suggesting that HvNAC013(177–346) is highly unfolded. Furthermore, NMR experiments in the interval from 20 to 200 MHz were identical and confirmed that the C-terminal domain of HvNAC013 exists predominantly as a monomer in solution (data not shown).

Structure formation in proteins with ID is typically studied as a function of solvent conditions (4), and TFE can be used to analyze secondary structure propensity (42). This additive was used to investigate if His6-HvNAC005(173–355) and HvNAC013(177–346) were prone to inducible structure changes using far-UV CD. For both proteins addition of TFE changed the global minimum from 200 nm, typical of an unfolded state, to 222 and 208 nm, characteristic of \(-\)helix structure (Fig. 7, A and B). In both cases, the \(-\)helix structure was adopted through intermediate structural stages. Whereas HvNAC013(177–346) changed the structure in the range of TFE concentrations used, His6-HvNAC005(173–355) reached equilibrium at a TFE concentration of 30% and did not gain additional structure by a further increase of the TFE concentration (Fig. 7, A and B).

Altogether, and in agreement with in silico analyses, the biophysical studies of the C-terminal domains of HvNAC013 and HvNAC005 showed that these are largely unfolded. The HvNAC013 TRD displayed a pre-molten globular state, yet both HvNAC005 and HvNAC013 C termini were able to adopt an inducible \(-\)helix structure by addition of TFE.

HvNAC013 TRD Interacts with the Promiscuous Plant-specific RST Domain of HvRCD1—None of the characterized NAC protein interactions involves the NAC TRD. However, it was recently reported that the Arabidopsis protein RCD1, which interacted with TFs from many different families, also interacted with the closest Arabidopsis homolog of HvNAC013, ANAC046 (20). The mutant rcd1 allele in Arabidopsis caused radical-induced cellular superoxide accumulation and cell lesion spreading (43). The promiscuity of RCD1 suggested that this protein interacts with structurally flexible TF regions such as TRDs making dissection of the interactions of great functional and structural interest. It was, therefore, obvious to test if HvNAC005 and HvNAC013, and in particular their flexible C termini, showed affinity for promiscuous RCD1.

A BLAST search using AtRCD1 as query revealed an orthologue in barley, named HvRCD1. From expressed sequence tag data, HvRCD1 was predicted to consist of 547 amino acid residues. However, the cDNA sequence isolated from barley leaves encoded a 579-amino acid residue protein corresponding in structure to AtRCD1 (supplemental Fig. S2). The proteins contain an N-terminal WWE protein-protein interaction domain followed by a poly(ADP-ribose) polymerase domain, and a C-terminal plant-specific RCD1, SRO, TAF4 (RST) domain (Fig. 8A). The RST domain was identified from RCD1-like proteins and TAF4, a component of the transcription initiation factor complex TFIID (20). The global sequence similarity
between HvRCD1 and AtRCD1 is 34% and an even higher degree of similarity was found for the conserved domains.

A directed yeast two-hybrid assay using full-length HvNAC013 and HvRCD1 as bait and prey, respectively, suggested that the proteins interacted (Fig. 8B). Similar results were obtained when HvRCD1 acted as bait and HvNAC013 as prey (Fig. 8C). Analysis of the ability of the isolated domains of HvNAC013 to interact with HvRCD1 showed that the interaction was mediated by the TRD of HvNAC013 (Fig. 8D). It was previously suggested that the RST domain was involved in interactions between RCD1 and target TFs (20). Therefore, the TRD of HvNAC013 and the RST domain of HvRCD1 (residues 485–579) were analyzed for the ability to interact. This revealed that the interaction was mediated by the unstructured TRD of HvNAC013 and the RST domain of HvRCD1 (Fig. 8D).

Recombinant versions of the HvNAC013 TRD and HvRCD1 RST domains, His6-HvNAC013(177–346) and GST-HvRCD1(485–579), were produced and used for in vitro pull-down assays (Fig. 8E). Western blot analysis showed that His6-HvNAC013(177–346) bound to immobilized GST-HvRCD1(485–579), but not to GST. This in vitro experiment confirmed the interaction between HvNAC013 and HvRCD1 and further confirmed that the TRD of HvNAC013 and the RST domain of HvRCD1 were sufficient for the interaction. HvNAC005 and HvRCD1 were also analyzed for interactions in yeast two-hybrid assays and in vitro pulldown assays. However, no interaction was detected for these proteins (Fig. 8F and G), which cannot be explained by a lack of DBD-HvNAC005(1–355) expression in yeast (Fig. 5E).

To extend our examination of structure-function relationships of the HvNAC013 TRD, we analyzed the truncations used for transcriptional analysis (Fig. 5) for the ability to interact with HvRCD1 (Fig. 8H). In contrast to full-length HvNAC013 and its TRD, HvNAC013(1–250), lacking the 96 C-terminal amino acid residues of HvNAC013, did not interact with HvRCD1. In contrast, mutation of the conserved aspartate (D217A) and leucine (L212A) of the LP motif had no effect on the interaction. In conclusion, the ability of the HvNAC013 TRD to interact with RCD1 resides within the 96 C-terminal amino acid residues of HvNAC013, and no interaction was detected between the LP motif, conferring transcriptional activity to HvNAC013, and HvRCD1 (Fig. 8H).

Fluorescence spectroscopy was also used to analyze the interaction between HvNAC013(177–346) and His6-HvRCD1(485–579). Protein-protein interactions can be followed by fluorescence spectroscopy because changes in tryptophan and tyrosine fluorescence indicate a change in the environment around the fluorophore. Only one tryptophan residue is present in the HvNAC013 and HvRCD1 domains at positions 344 and 492, respectively. HvRCD1(485–579) has one tyrosine residue at position 510, whereas six tyrosine residues are present in HvNAC013(177–346), of which five are among the last 96 amino acid residues. The proteins were excited at either 280 or 296 nm and emission scans between 310 and 400 nm were recorded. Spectra of individual proteins and mixed HvNAC013(177–346) and His6-HvRCD1(485–579) were recorded (Fig. 9, A and B). The sum of the spectra of individual proteins was used as reference for noninteracting proteins. \( \Delta \lambda_{\text{max}} \) was calculated and a minor change of 4.5 and 3.0 nm in fluorescence \( \lambda_{\text{max}} \) upon mixing of the two proteins was observed from the excitation wavelengths. This suggested that the environment around the aromatic residues changed to more hydrophobic upon interaction (Fig. 9B). This can be explained by a direct involvement of one or several tryptophan and tyrosine residues. Three of the tyrosine residues are located within the YF and RR motifs of HvNAC013 (Fig. 9B).

It is the general notion that many ID proteins fold upon binding to their protein interaction partners (4). However, more transient and flexible interactions may also occur that are not accompanied by structural changes of either partner (44). CD was used to analyze the structural changes occurring upon interaction further (Fig. 9, C and D). As the structure of the RST domain was unknown, a CD spectrum of His6-HvRCD1(485–579) was recorded. The pronounced minimum at 222 and 208 nm suggested that the domain is composed mainly of \( \alpha \)-helical structures (Fig. 9C), in accordance with secondary structure predictions (supplemental Fig. S2). The CD spectrum of interacting HvNAC013(177–346) and His6-HvRCD1(485–579) seemed to be dominated by the HvNAC013 structure (Fig. 9D). A change in absorbance at both 222 and 208 nm was observed for mixed compared with individual proteins suggesting a shift toward a
more unfolded state or more extended structure within the complex (Fig. 9D). This is a strong indication that HvNAC013 and HvRCD1 do not interact through a coupled binding and folding mechanism, but rather through a slight reorientation of the conformational ensemble of either or both proteins, possibly involving extended structure formation.

**HvRCD1 Interacts with HvNAC013 and Other TFs through Their ID Regions**—To test the hypothesis that RCD1 can interact with many structurally different TFs because of their structural flexibility, the interaction between AtRCD1 and previously reported interaction partners AtbHLH011 and AtMYB91 (20) was analyzed further using two-hybrid assays. These proteins represent major plant TF families and are characterized by a basic helix-loop-helix or SANT (Swi3, Ada2, N-CoR, TFIIIB) DBD and contain regions with predicted ID (Fig. 10A and supplemental Fig. S3). Both full-length proteins and the regions with ID were examined for interaction with AtRCD1. This confirmed the interaction between AtRCD1 and the two proteins (Fig. 10B). Furthermore, the ID region of AtbHLH011 and the C-terminal ID region of HvNAC013 served as negative controls. Input, total protein added to the glutathione beads; last wash, the volume was equal to the elution volume. GST and GST-HvRCD1(177–346) were indicated with arrows. E, GST pulldown assay of the HvRCD1 RST domain and the HvNAC013 TRD. GST and GST-HvRCD1(177–346) using antihistidine and GST antibodies. Glutathione-Sepharose beads incubated with His$_{6}$-HvNAC013 and GST and His$_{6}$-HvNAC013 served as negative controls. Input, total protein added to the glutathione beads; last wash, the volume was equal to the elution volume. GST and GST-HvRCD1(177–346) were indicated with arrows. F, GST pulldown assay of the HvRCD1 RST domain and the HvNAC005 C-terminal domain (residues 173–355). Conditions as described in E. G, directed yeast two-hybrid assay between HvNAC005 and HvRCD1. Conditions are as described under B–D, but without 3-amino-1,2,4-triazole. H, directed yeast two-hybrid assay between HvNAC013 and HvRCD1. Conditions are as described under B–D, but with 90 mM 3-amino-1,2,4-triazole to inhibit self-activation. PARP, poly(ADP-ribose) polymerase.
DISCUSSION

In this study we present a comparative structure-function analysis of two distant NAC TFs associated with senescence. Both HvNAC013 and HvNAC005 have a prototypical NAC DBD and bound the NACBS with affinities typical of NAC TFs (8) suggesting that they mediate function through binding to NACBSs in target genes. They also both have C-terminal domains dominated by ID, typical of TRDs (5), but only HvNAC013 activated transcription. Thus, despite structural similarities, HvNAC013 and HvNAC005 could have different functionality in the same physiological context. Although most NAC TFs are transcriptional activators (8, 37) some, e.g. Arabidopsis CALMODULIN BINDING NAC and VND-INTER-ACTING2 (VNI2), function as repressors (9, 45). VNI2 inhibited transcriptional activation by VND TFs involved in xylem vessel formation, possibly by forming inactive heterodimers with the VND proteins, and had intrinsic repression activity typical of an active repressor (9). It is not possible to predict if HvNAC005 is a repressor. It will be interesting to test if HvNAC005 functions as a repressor modulating activity of NAC TFs involved in senescence through heterodimerization, in analogy to the mechanism suggested for VNI2 (9), or hinders activity of NAC TFs by competitive binding to NACBSs.

As shown in this study several motif-containing regions contribute to transcriptional activity of the TRD of HvNAC013. The LP motif was sufficient for activity, and mutational changes of single amino acid residues in this motif resulted in decreased activity. Removal of the complete motif from the full-length protein may have an even larger effect on activity, because it...
contains several highly conserved amino acid residues likely to be of functional importance. The motif is frequent in Arabidopsis NAC TFs (identified in 28 of 109 NAC TFs) and positioned at the border between order and disorder in both HvNAC013 and the Arabidopsis NAC TFs (8). Transcriptional activity, evolutionary conservation and proliferation, and conservation of the order-disorder prediction for the LP motif region suggest that it plays an important functional role in NAC TFs. In ANAC012, involved in xylem fiber development, this motif was not able to activate transcription. This activity was instead mediated by the WQ motif, not found in HvNAC013 (37), demonstrating complexity of the NAC TRDs. Analysis of both HvNAC013 and ANAC012 (37) transcriptional activity was performed in yeast. An even more complex in planta picture, involving specific and general TFs and cofactors, can be expected. Based on knowledge from well studied TFs, such as p53, for which many interaction partners binding to ID regions have been identified (39), the NAC C-terminal domains can be expected to participate in interactions with numerous protein partners. Here, we have initiated a systematic analysis of one of the complex NAC TRDs and show that a specific sequence motif is functionally important, possibly by interacting with a specific protein partner.

The biophysical analysis of HvNAC013 and HvNAC005 showed that ID dominates their C-terminal domains, although both predictions and CD analyses suggested local and highly limited formation of secondary structures (Figs. 4 and 6, A and B). Although RCD1 was identified as a potential regulator of NAC TFs (20), interactions between RCD1 and NAC TFs have not been characterized in details. The indispensability of the 96 C-terminal amino acid residues of HvNAC013 for the interaction with HvRCD1 together with the predicted structure for the last 24 residues of HvNAC013 (Fig. 4) may suggest that a MoRF is present in this part of HvNAC013 (6, 7). However, analysis of the HvNAC013-HvRCD1 interaction by fluorescence spectroscopy and CD did not suggest significant folding upon interaction. In contrast, the small structural changes observed may suggest redistribution of the conformational ensemble suggestive of formation of a more extended conformation upon interaction. Lack of folding upon binding is also the case for protein interactions involving, e.g. the intrinsically disordered z chain of the T-cell receptor (46). Thus, fuzziness, defined as disorder in the partner-bound state (44), may also characterize the interaction of the HvNAC013 TRD with RCD1, although atomic resolution structural information is needed to conclude this.

Arabidopsis RCD1 can interact with such different proteins as viral movement protein from turnip crinkle virus (47), the salt tolerance pathway component Salt Overly Sensitive (48), and different TFs (20). As shown here, the barley RST domain is sufficient for the interactions with TFs. This domain was identified from RCD1-like proteins and TAF4, which is involved in assembly of the general TF complex TFIID (49). This suggests that RCD1 is a regulator of TFs. Very interestingly, HvNAC013, but not HvNAC005 for which no activation activity was assigned, interacted with HvRCD1. The promiscuity of RCD1 interactions has not been explained previously. Here we show that barley and Arabidopsis RCD1 interact with TF regions dominated by ID, which may allow for flexibility in the interactions. Secondary structure predictions (supplemental Fig. S2) and CD analysis (Fig. 9C) suggested that the RST domain forms an a-helix fold that would make a solid platform for multiple, regulatory interactions with partner proteins. Thus, RCD1 could possibly be a novel hub. Hub proteins are divided into party hubs, which have multiple, simultaneous interactions, and date hubs with multiple, sequential interactions (50). Date hubs have been associated with both ID and transient binding (51). In the case of RCD1 and the TF interactions, ID is associated with the TFs, and the RST domain could be a folded date hub domain.

14-3-3 proteins, involved in crucial processes such as mitogenic signal transduction, cell cycle control, and apoptosis, are also folded hubs (52). More than 200 proteins, representing a diverse array of signaling proteins and receptors, interact with 14-3-3 proteins. Structure analysis of complexes between a 14-3-3 protein and peptides from different interaction partners showed that the broad specificity could be explained by a broad binding site allowing binding of different peptides. 14-3-3 recognition most likely involves coupled binding and folding of the recognition region (52). Like the RST domain of RCD1, 14-3-3 hub proteins have an a-helix structure, and interactions between the RST RCD1 domain and different TFs may have other similarities to interactions involving 14-3-3 proteins.

Further analyses should address if different TFs use common LMs (7) in binding to RCD1. Interestingly, HvNAC013 shares a 23-amino acid residue motif (supplemental Fig. S1, residues 296–318), positioned at the border of the predicted disorder to order transition in the binding region (Fig. 4), with Arabidopsis ANAC046, ANAC087 ANAC079/80, and ANAC100. This motif, and the RR motif, which displays significant a-helix propensity (data not shown) suggestive of a MoRF (6), should be analyzed further for a functional role in interaction with RST domains. AtRCD1 was more promiscuous in its interactions with TFs than its parologue SRO1, which also contains an RST domain (20). It will also be interesting to analyze HvNAC013, HvNAC005, and other NAC TFs for interactions with the RST domain protein TAF4 as a direct link to transcriptional core components. Another TFIIID component, TAF9, interacts functionally with just a 5-amino acid residue motif (FSDLW) of p53 (3) making even small motifs of interest to binding.

The physiological relevance of the interaction between the TFs and RCD1 remains elusive. In Arabidopsis, RCD1 plays multiple roles in both stress and reactive oxygen species responses, and development (19, 20), and the rcd1 mutant plant displayed several pleiotrophic phenotypes including senescence. Despite having a polyl(ADP-ribose) polymerase domain RCD1 does not have polyl(ADP-ribose) polymerase enzymatic activity (53), and the molecular function of RCD1 remains unclear. Although HvRCD1 was expressed during senescence, no significant induction of the expression was seen during senescence (data not shown), and AtRCD1 also exhibited only subtle regulation in response to stress exposure (53), suggesting that the physiological functions of RCD1 is not reflected at the transcriptional level. In summary, RCD1 and its relatives are likely to play broad physiological roles through regulation of different TFs. ANAC046, the Arabidopsis NAC most closely related to HvNAC013, was one of only three NAC TFs that
interacted with RCD1 (21). This together with conservation of the interaction suggest that the biochemical interaction between HvNAC013 and HvRCD1, here described in mechanistic details, is of relevance to HvNAC013 and a role in plant senescence.

Acknowledgements—We thank Maria Joergensen and Charlotte O'Shea for valuable technical assistance. We thank Dr. Colin Gordon,

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