HRT, a Novel Zinc Finger, Transcriptional Repressor from Barley*

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A barley gene encoding a novel DNA-binding protein (HRT) was identified by southwestern screening with baits containing a gibberellin phytohormone response element from an α-amylase promoter. The HRT gene contains two introns, the larger of which (5722 base pairs (bp)) contains a 3094-bp LINE-like element with homology to maize Colonist1. A gibberellin phytohormone (GA)1 mediate seed germination, cellular and vegetative growth, and flower and fruit development (1, 2). Genetic analyses of GA-dependent processes in Arabidopsis have identified potential GA-response regulatory genes (3–7). Biochemical studies of germination in cereal aleurone cells indicate that cGMP and Ca2+ act as second messengers in GA-dependent signaling pathways in which heterotrimeric G proteins and a protein phosphatase may participate (8–11).

Molecular studies have delineated cis-acting elements in promoters of GA-regulated, cereal α-amylase genes, including GA response elements (GARE) (12–14) which function with coupling elements in GA response complexes (GARC) (15). These elements have been used to characterize DNA-binding proteins which may be involved in the activation of gene transcription in response to GA (16, 17). Rushton et al. (18) used the Opaque-2-like coupling element from a low pi, Amy2/3 α-amylase gene promoter as a bait in southwestern screens to isolate ABF1 and -2, although functional analyses of their roles in regulating α-amylase transcription have not been reported. Gubler et al. (19), noting that a portion of the GARE consensus (TAACAAR, R = G or A) may be a Myb-binding site, isolated a GA-responsive Myb protein (GAMyb). They showed that GAMyb mRNA accumulates earlier than that of α-amylase, that the protein specifically binds functional GAREs in vitro, and that overexpressed in transient assays, activates transcription in vivo from an Amy1 promoter in cells not treated with exogenous GA.

Interestingly, GAMyb mRNA accumulation is super-induced by cycloheximide (19) suggesting that a short-lived repressor activity is involved in early responses to GA. Several genes have been cloned that may negatively regulate GA signaling pathways. Maize VP1 has been shown to act both as a transcriptional activator of seed maturation genes and also as a repressor, either directly or indirectly, of germination-specific genes such as α-amylase (20). Arabidopsis SPY (4), a tetratricopeptide repeat protein, and GAI (6) and RGA (7), both putative transcription factors, are also thought to negatively regulate GA responses.

Our interest in the mechanisms of gene expression in cereals prompted us to search for additional DNA-binding regulatory proteins. To this end, we southwestern-screened barley aleurone cDNA libraries using GARE-containing baits from the high pi, Amy1/6-4 promoter, which lacks an Opaque-2-like coupling element. Here we report the results of cloning, expression, and structure/function analyses of a novel protein referred to as HRT (Hordeum repressor of transcription).

EXPERIMENTAL PROCEDURES

Plant Material—Aleurone layers were prepared from grains of barley (Hordeum vulgare cv. Himalaya, 1985 harvest, Dept. of Agronomy, Washington State University, Pullman, WA) and treated with hormone as described previously (13, 21). Vegetative and floral tissues for RNA extraction were dissected from 6-week-old, flowering Himalaya plants grown at 15 °C in a greenhouse. Tissues for RNA and DNA extraction were frozen in liquid N2 and stored at −80 °C.

Isolation and Analysis of RNA, cDNA, and Genomic Clones—RNA for Northern blotting was isolated, separated in formaldehyde gels, blotted to nitrocellulose, and hybridized to random-primed cDNA probes after standard protocols (22, 23). Probes for Northern hybridization were HRT, a high pi Amy1 cDNA (pMC) (24), the C terminus of GAMyb previously shown to be a gene-specific probe (19), and a wheat rRNA clone (25). Blots were stringently washed at 65 °C in 0.1 × SSC, 0.1% SDS for 30 min prior to exposure. mRNA for cDNA library construction was isolated as described previously (21). Pooled mRNA templates for cDNA synthesis were extracted from aleurone layers incubated with or without 2 μM GA and/or 20 μM cycloheximide for 2, 6, 12, and 24 h. cDNA was synthesized by the RNase H method (26), ligated to EcoRI adaptors, and cloned in Agt11. 4 × 106 primary recombinants were screened by southwestern blotting using a denaturation/renaturation cycle (27). Two GARE DNA-binding site probes were used together, one containing 2 copies of the Amy1/6-4 189 to −120 region, the other 8

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§ The abbreviations used are: GA, gibberellic acid 3; ABA, abscisic acid; PAF, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; NLS, nuclear localization signal; GUS, β-glucuronidase; bp, base pairs; kb, kilobase pairs; GARE, GA response elements; GARC, GA response complexes; GAMyb, GA-responsive Myb protein; PCR, polymerase chain reaction; DAPI, 4′,6-diamidino-2-phenylindole.
copies of the −148 to −128 regions (12). A >1.5-kb size-selected cDNA library made from germinating Himalaya barley seeds, kindly provided by Dr. Rob Schuurink (Max-Planck-Institute, Cologne), was screened with the HRTb cDNA probe to isolate full-length clones. 500 µg of genomic DNA was isolated from 20 g of 9-day-old, etiobased barley aleurone cells from immature embryos from crosses with 1% cetvlyl-rimethylammonium bromide (28). Two genomic libraries were constructed in λZAPI (Stratagene), one with DNA completely digested with SacI, the other with DNA partially digested with Sau3A. Phages were plated on Escherichia coli ER1647 (MerA- and MerB-, New England Biolabs) and screened with random-primed HRTd cDNA probe. DNA sequencing of both strands was performed at least on a double-stranded template with Sequenase (U. S. Biological Corp.) and autoradiography or with dye primers and PCR (Thermo sequenase, Amersham) and analyzed on an Applied Biosystem 373 DNA sequencer. DNA sequences were analyzed using programs in the GCG package (29).

DNA Constructs—Binding probes for southwestern and gel mobility assays are shown in Fig. 2C. They were labeled to a specific activity of approximately 1 × 10^6 cpm/µg and included single and multimeric copies of the Amy1/6-4 GARC (70 bp; −189/−120), GARE (21 bp; −148/−128) and T- or pyrimidine box (21 bp; −177/−175), and of the rab16A 2-bp dyad symmetry site (21 bp; −215/−195) (20). The HRTd cDNA in pSK was excised and circularized from λZAPI according to standard protocols (21). The coding region for the cDNA was amplified by PCR using linker-primers containing an optimal translation start codon. The cDNA was inserted into the pET (Novagen) expression vector TOPO vector and sequenced to confirm the mutations. PCR products were digested with EcoRI/XhoI and directionally-inserted into pSK (Stratagene) and pGEX-1T (Pharmacia). In vitro mutagenesis to produce mutated HRTd3 polypeptides was performed according to Kunkel (30) using single-stranded pSK containing the EcoRI/XhoI fragment of HRTd3 (above) and the following primers: C500S, 5′-GCCTCAGC-3′; C510S, 5′-GGTGCCTCG-3′; C520S, 5′-GATGTTATCCCTGTCGGAACCTAC CCTG-3′; C531S, 5′-CAGAGAGAAGAATCTGCTGCAACAA-3′; HS54A, 5′-AGAAGA AACAGAGAATCTGCTGCAACAA-3′; C540A, 5′-AATGTTATCCCTGTCGGAACCTAC CCTG-3′; C550S, 5′-CTCGAGTCTCGAGTCGA-3′. The double mutant C500S/C510S was generated by the standard single-stranded plasmid containing the mutation C510S with primer (C500S). The resultant mutant HRTd3 fragments were directionally inserted into the EcoRI/XhoI sites of pGEX-4T. All constructs were sequenced to confirm the mutations.

The HRT/GUS fusions for nuclear localization (Fig. 4) were made by PCR amplification using linker-primers containing an optimal translation start (31). The products were inserted into the EcoRi/SmaI sites in expression vector pABJ188, a derivative of pFMG (32). The 02/GUS control was B223–254:GUS (32). The GUS control was made by replacing the 02/GUS fusion in B233–254:GUS by GUS from pBI101.1 (CLONTECH) using BamHI/SacI. For expression assays in aleurone cells an effector control was made by creating the 3′ splice site of the ubiquitin intron 1 by first cleaving the maize ubiquitin promoter/intron from pAHC18 and using this primer to insert the 5′ splice site of the maize ubiquitin promoter/intron as an EcoRI/XhoI fragment into the pET (Novagen) expression vector pGEX-4T. All constructs were sequenced to confirm the mutations. The HRTd effector was made by PCR amplification using BglII or BamHI linker-primers containing in-frame translation initiation sequences or the SV40 NLS and a stop codon as required. The amino acid residues included in these polypeptides are noted in Figs. 5 and 6. The products were inserted into the BamHI site of pAhCh183 (33) from which the luciferase coding sequence had been removed by restriction with BamHI. The resulting plasmids contained the UbI promoter transcriptionally fused to HRT sequences followed by the NOS terminator.

RESULTS

Production and Analysis of GST/HRT Fusion Proteins—GST/HRT fusion proteins were expressed in E. coli VL21 cells after induction with 0.5 mM isopropyl-1-thio-β-D-galactoside for 3 h at 37 °C. Proteins were extracted with sonication in 1/20 volume buffer (25 mM HEPES/KOH, pH 7.9, 1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 0.05% Nonidet P-40, 0.5 µg/ml poly(dI:dC) 22,000 cpm radioactive probe (approximately 0.5 ng) end-labeled with [35S]DATP Klenow enzyme and approximately 100 ng of the purified HRTdb1, HRTdb3, or HRTdb2 and -3 GST fusion proteins. Reactions, with or without 5- or 50-fold unlabeled competitor DNAs, were incubated at room temperature for 30 min, and 10 µl were then analyzed by electrophoresis on a 5% acrylamide gel (75:1) in 1× high ionic strength electrophoresis buffer (23) at 220 V at 4 °C. Gels were dried after electrophoresis and autoradiographed. Zinc blot assays with 65ZnCl2 was performed according to Schiff et al. (34).

Transgenic Gene Expression Assays in Onion Epidermal and Barley Aleurone Cells—Cells in the epidermal layer of onion bulbs were transformed by Agrobacterium tumefaciens as described by Varagona et al. (32). After bombardment, cell layers were incubated for 16 h at 21 °C in darkness. Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the GUS substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid to localize nuclei and GUS reporter activity (35).

The GUS constructs used and procedures for assaying GUS reporter expression and firefly luciferase internal standard activities have been described previously (35, 36, 37). The promoters used to drive GUS expression included Amy1/6-4, Amy2/32b, an Amy2/32b mutant with an ABRE replacing the GARE, the CaMV 35 S promoter/Sh1 intron, and the maize Adh1 promoter/intron construct from pmspld pBARGUS (38). The firefly luciferase reporter under control of the maize ubiquitin 1 promoter with first intron (Ubi/Luc) was included as an internal standard to correct for transfection efficiencies. Luciferase values for the HRT sets were multiplied by the control/test ratio from each experiment to correct for the repressive effect of HRT (Fig. 5). To exclude the possibility that the repressive effects of full-length HRT were due to an artifact involving the use of the Ubi/Luc internal standard, an experiment without an internal LUC standard was performed in which the Amy1/6-4 promoter/GUS reporter was expressed in GA-treated aleurone layers only in the presence of either the effector control or the full-length HRT test construct (n = 24 for each). This indicated that HRT reduced the level of GUS expression to 25% of the control (p = 0.000012; data not shown). This level of repression was the same as that obtained when the Ubi/Luc internal standard was used and validates the correction of those values in the experiments shown in Fig. 5. The reporter constructs used in the solution to coat the tungsten particles were: pAHCh18 (Ubi-Luciferase-NOS; Ref. 39), 5 µg, Ubi-HRT effector constructs, 10 µg, and α-amylase promoter/intron-GUS reporter constructs, 10 µg, all in a total volume of 30 µl. Statistical comparison between sets was performed by a two-tailed Student’s t test.

Isolation and Characterization of HRT cDNAs and Corresponing Gene—Two cDNAs (Fig. 1A; HRTa, 358bp, and HRTb, 790 bp) were initially isolated by southwestern screening of barley aleurone cDNA libraries (27). The cDNAs were synthesized with pooled poly(A)+ RNAs from aleurones incubated with and without GA or cycloheximide for between 2 and 24 h. Such RNA populations should encode various proteins that may be involved in transcriptional regulation of α-amylase and other genes (13, 19). The baits used were two copies of the 70-bp GARC region (−189 to −120) and six copies of the 21-bp GARE region (−148 to −128) of the Amy1/6-4 promoter (12). A longer (HRTc, 1302 bp) and two full-length clones with the same 5′ nucleotide and different polyadenylation sites (HRTd, 1928 bp, and HRTe, 1836 bp) were isolated by hybridization to a size-selected (>1.5 kb) cDNA library with HRTb as probe. Complete sequencing of both strands of all clones revealed that they contain identical, overlapping sequences encoding the same protein (Fig. 1A). The C-terminal polypeptides encoded by HRTa and -b were in frame with β-galactosidase (β-Gal), while HRT-c-e were not. These results indicate that the HRTa and HRTb C-terminal polypeptides contain a DNA-binding domain(s).

Rabbit polyclonal antibodies were raised against a fusion protein between E. coli GST and the HRTb ORF. That HRTd and -e encode a full-length ORF was confirmed by immunoprecipitation experiments which showed that the protein produced by in vitro transcription/translation of HRTd had the same mobility in SDS-PAGE as that produced by in vitro translation of poly(A)+ RNA isolated from barley aleurone (data not
The HRT protein of 548 amino acids has a calculated molecular mass of 60.3 kDa and a pI of 9.8. It contains two putative NLS sequences (Arg276–Arg292 and Arg527–Arg530), while similar amino acid residues are underlined. The HRT protein of 548 amino acids has a calculated molecular mass of 60.3 kDa and a pI of 9.8. It contains two putative NLS sequences (Arg276–Arg292 and Arg527–Arg530), while similar amino acid residues are underlined.

Consensus sequences occur in at least six of these. In the consensus, identical residues in two of the sequences are capitalized while similar residues are stippled. Intron sequences, diagnostic of non-long terminal repeat retrotransposable elements, are introduced. One probable frameshift, near nt 703 of Y10013, produces a C-terminal region containing four putative zinc fingers (Fig. 1; Y10013db1–4) similar to HRTdb1–3. Regions of the N terminus of the Arabidopsis protein, and most of the Vicia protein, are also similar to HRT. The Vicia C-terminal region contains a putative, bipartite NLS sequence and two putative zinc fingers (Fig. 1; X97909db1 and -2).

The HRT gene sequence data have been submitted to the DDBJ/EMBL/GenBank™ data bases under accession number AJ001317. The complementary strand of the second, large intron (5722 bp) contains a 3094-bp sequence with homology to the maize long interspersed nuclear element or LINE-like retrotransposon sequence Colonist1 (41). The barley sequence is flanked by 18-bp direct repeats diagnostic of non-long terminal repeat retrotransposable elements. Both this barley element and Colonist1 may contain frameshifts C-terminal to the reverse transcriptase open reading frame and lack adenine-rich termini. It is therefore difficult to determine whether they represent full-length elements. We conclude that the second intron of HRT contains a LINE-like element whose distribution and activity in the barley genome can now be studied (43).

**Fig. 1.** A, comparison of the predicted protein products encoded by HRT (top), V. faba cDNA (accession number X97909), and A. thaliana cDNA (accession number Y10013; bottom). In the consensus, identical residues in two of the sequences are capitalized while similar amino acid residues are lowercase. The first amino acid residues of the HRTa–e cDNAs are noted above the sequences. Putative DNA-binding domains are underlined, and predicted nuclear localization signals stippled. Consensus residues occur in at least six of these. Data base searches revealed that HRT is similar to proteins encoded by cDNAs isolated from seeds of Arabidopsis thaliana (accession number Y10013) and Vicia faba (accession number X97909), but no significant homology to other proteins was found. Sequence alignments with HRT indicated that the Arabidopsis cDNA probably does not contain a full-length ORF, even if frameshifts are introduced. One probable frameshift, near nt 703 of Y10013, produces a C-terminal region containing four putative zinc fingers (Fig. 1B; Y10013db1–4) similar to HRTdb1–3. Regions of the N terminus of the Arabidopsis protein, and most of the Vicia protein, are also similar to HRT. The Vicia C-terminal region contains a putative, bipartite NLS sequence and two putative zinc fingers (Fig. 1B; X97909db1 and -2). HRT contains recognition sites for protein kinase activities (40), although these sites do not appear to be conserved in the Arabidopsis or Vicia proteins. These comparisons indicate that the three proteins contain regions of similar sequence, including the repeated, putative DNA-binding DNA-binding domains with the consensus VCGXDGX2CX3PVX2RKRCX2HKG (Fig. 1B, below).

**HRT**

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region of the Amy1/6-4 GARC (Fig. 2B). The specificity of this binding was examined following the addition of unlabeled, competitor DNAs of approximately the same size as the labeled probe. This showed that a 50-fold excess of four copies of a 21-bp region of the Amy1/6-4 promoter containing only the GARE fully competed for binding to the GARC. Gel mobility assays also demonstrated that fusion proteins containing HRTdb3 (Ser500–Lys548) bound the same GARE-containing probe, indicating that a single HRT DNA-binding domain can bind DNA (Fig. 3A, lanes 1–4). In vitro mutagenesis was performed with the HRTdb3 fusion to identify amino acid residues important for DNA binding (Fig. 3A, lanes 5–12). In addition to the three Cys and one His residue conserved in all of the putative DNA-binding domains of HRT and the Arabidopsis and Vicia proteins, the HRTdb3 fusion contains one N-terminal and two C-terminal His residues conserved in all of the putative DNA-binding domains of HRT and the Arabidopsis and Vicia proteins.

**HRT Is Targeted to Nuclei—**To determine whether the two predicted NLS sequences (Arg276–Arg292 and Arg427–Arg520) in HRT can mediate import of the protein to nuclei, HRT fusions were introduced into onion epidermal cells by particle bombardment, and the expressed proteins were

![Fig. 2. HRT C-terminal regions bind Amy1/6-4 GARC sequences. A, southwestern assay with plaque-purified phage demonstrating DNA binding by fusion β-Gal/HRTdb cDNA (Lys397–Lys548). Probes were (clockwise): 6′ x rab16A ABRE, 2 x rab16A ABRE, 4′ x Amy1/6-4 GARE, 4′ x Amy1/6-4 T-box, 2 x Amy1/6-4 GARC, 1 x Amy1/6-4 GARC. B, gel mobility assay with purified GST protein (lane 1) and GST/HRTdb2 and -3 fusion proteins (lanes 2–7) demonstrates that GST/HRTdb2 and -3 bind Amy1/6-4 GARC sequences (lane 2) and that binding is competed by Amy1/6-4 GARE (lanes 3 and 4), but not by Amy1/6-4 T-box (lane 5), rab16A ABRE, or ABRE (lanes 6 and 7) sequences. C, GARC, GARE, ABRC, and ABRE sequences used in A and B above (see also “Experimental Procedures”).

![Fig. 3. DNA binding is mediated by zinc finger domains. A, gel mobility assay demonstrating that HRTdb1 binds DNA, and the effects of mutations within the HRTdb3 DNA-binding domain on the ability of purified GST/HRTdb3 fusions to bind a probe containing three copies of the Amy1/6-4 GARC region. B, Coomassie-stained SDS-PAGE (left) and protein blot probed with radioactive 65Zn2+ (right) demonstrating that GST/HRTdb3 (10 μg) binds Zn2+. Controls are GST (1 μg) alone and the zinc-binding proteins alkaline phosphatase (10 μg) and carbonic anhydrase (1 μg). Cys residues (Cys500, Cys540, and Cys541; Figs. 1A and 3A). Mutagenesis of two of these (Cys500 and Cys540) or of nonconserved Ala512, did not affect DNA binding (Fig. 3A, lanes 4 versus 5, 8, and 12). However, mutagenesis of Cys510 severely reduced binding, while mutagenesis of Cys500, Cys531, or His534 abolished binding (Fig. 3A, lanes 6, 9, 10, and 11). Double mutagenesis of both Cys500 and Cys540 also abolished binding (Fig. 3A, lane 7), suggesting that Cys500 may partially compensate for Cys510 in mutant HRTdb3 polypeptides. Zn2+-binding assays, which included the zinc-binding enzymes carbonic anhydrase and alkaline phosphatase as well as GST as controls (34), showed that HRTdb3 binds Zn2+ (Fig. 3B). This binding could be abolished by competition with cold Zn2+ or Cd2+, but not Mg2+, indicating specificity for group 2b ions (data not shown). These results indicate that HRTdb3, and presumably HRTdb1 and -2 and the similar regions in the Arabidopsis and Vicia proteins, represent a novel zinc finger DNA-binding domain.
localized as described by Varagona et al. (32). The 68-kDa GUS protein was detected in the cytoplasm of plant cells because it lacks an NLS and is too large to move passively into nuclei (Fig. 4). A GUS fusion protein containing HRT amino acid residues Ala<sup>270</sup>–Lys<sup>548</sup> was targeted to nuclei, while a fusion containing residues Lys<sup>379</sup>–Lys<sup>548</sup> was not. This indicates that the basic sequence RRKR (residues 527–539) is insufficient to direct nuclear import of the fusion protein, while the bipartite, basic sequence KRR<sup>292</sup>SEGYKVKKIDVI<sup>301</sup> may be an NLS. A GUS fusion containing this bipartite sequence (Ali<sup>270</sup>–Ala<sup>301</sup>) was localized to nuclei, as was a fusion containing the NLS of the maize Opaque-2 b-ZIP protein used as a positive control (32). This and additional data presented below (Fig. 6) indicate that residues Arg<sup>276</sup>–Arg<sup>292</sup> function as an NLS and that HRT is targeted to nuclei.

**HRT Represses Transcription from Some Promoters**—To examine whether HRT affects transcription from promoters in plant cells, full-length or truncated HRT sequences were transcriptionally fused to the Ubi promoter (Fig. 5; Ref. 39). Particle bombardment was used to co-transfect these HRT effectors with GUS reporters into aleurone cells as described previously (13, 15, 36). All experiments included an effector control modified so as not to express HRT protein (see “Experimental Procedures”). The luciferase reporter under control of the maize ubiquitin promoter was localized to nuclei, as was a fusion containing the NLS of the constitutive maize Adh1 promoter (D, Amy2/32b promoter in which the GARE was replaced with an abscisic acid response element (ABRE, Ref. 15) (E). Results are presented as relative GUS expression, where the level of expression from each promoter in the absence of HRT is assigned a value of 1.0 so that the effects of HRT expression can be more readily evaluated. Absolute levels of expression from the different amylase promoter constructs have been published (36, 37); the level of 35 S/GUS expression was similar to Amy1/GUS, while the level of Adh1/GUS was ~33% of that value. GA treatments (2 µM) and ABA treatments (20 µM) are indicated.

**Expression of full-length HRT** markedly reduced expression of the Ubi/Luc internal standard to 25% of control. Accordingly, luciferase values for the HRT sets were multiplied by the control/test ratio from each experiment to correct for the repressive effect of HRT (Fig. 5; see “Experimental Procedures”). The effects of full-length HRT on transcription from different promoters are shown in Fig. 5. It can be seen that transcription from the high pi Amy1/6-4 promoter was induced 79-fold by GA in the presence of the effector control. In contrast, the GA-induced level of expression from the Amy1/6-4 promoter in the presence of HRT was only 22% of control (Fig. 5B). Similarly, while expression from the stronger, low pi Amy2/32B gene promoter was induced 78-fold by GA in the presence of the effector control, HRT reduced the GA-induced level of expression from this promoter by 61% compared with the effector control (Fig. 5B). Similar effects were seen with the constitutive CaMV 35 S promoter, such that HRT lowered the level of expression to 33% of that value. GA treatments (2 µM) and ABA treatments (20 µM) are indicated.
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HRT Truncations Containing DNA-binding Domains and an NLS Activate Transcription from Some Promoters—Truncated forms of HRT were further tested as effectors to determine whether nuclear localization is required for HRT activity in vivo, and whether the domain(s) of the protein responsible for its repressive activity are separable from the HRTdb1–3 DNA-binding domains. For example, HRTΔ1 contains approximately the C-terminal half of HRT including the functional NLS and the DNA-binding domains (Fig. 6, top). In contrast, HRTΔ2 lacks the NLS and HRTdb1. To facilitate the import of HRTΔ2 to the nucleus (Fig. 4), the simian virus 40 (SV40) T-antigen NLS (PKKKRKV; Ref. 31), which is known to function in plant cells, was attached to the carboxyl terminus of HRTΔ2 to form HRTΔ2-NLS.

Reporter assays indicated that HRTΔ1 increased the level of expression from the Amy1/6–4 promoter in aleurone layers not treated with hormone and slightly reduced the level in layers treated with GA (Fig. 6A). In contrast, HRTΔ2 did not affect reporter expression compared with effector controls (data not presented). However, HRTΔ2-NLS raised the level of expression in control aleurone layers not treated with GA to close to that obtained in layers treated with GA (Fig. 6A). Further N-terminal truncations of HRT exhibited similar effects. HRTΔ3-NLS, containing only HRTdb2&3, increased reporter expression in the absence of hormone to at least the level in the presence of GA but did not significantly affect the level of expression in the presence of GA compared with controls. HRTΔ4+NLS, which contains only HRTdb3, had approximately the same effect as HRTΔ2-NLS. However, HRTΔ4M-NLS, in which an amino acid residue within the zinc finger motif essential for DNA binding had been mutated (Fig. 3A; Cys531 → Ser), did not significantly affect reporter expression. These results indicate that polypeptides lacking approximately the N-terminal half of HRT do not mediate transcriptional repression but rather activate transcription. This activity is dependent upon the presence of DNA binding and nuclear localization domains. As HRTΔ4M-NLS does not significantly affect reporter expression, the SV40 NLS, while apparently responsible for localization, does not directly mediate the effects of the truncated HRT polypeptides on reporter expression.

A similar effect of HRTΔ2-NLS, although of lesser magnitude, was seen with the Amy2/32b promoter/GUS reporter (Fig. 6B). In this case, HRTΔ2-NLS increased reporter expression in the absence of hormone, with no significant effect on the level of expression in GA-treated layers. HRTΔ2-NLS also strongly increased expression from the constitutive CaMV 35 S promoter (Fig. 6C), although it did not affect transcription from the maize Adh1 promoter/GUS reporter (Fig. 6D). These effects are consistent with those seen with the same promoters and the full-length HRT effector (above). They suggest that the HRT N-terminal region mediates transcriptional repression.

A HRT truncation lacking the HRTdb1–3 DNA-binding domains (Fig. 6; HRTΔ5) was used in initial attempts to localize the HRT domain(s) responsible for transcriptional repression. This C-terminally truncated polypeptide did not significantly affect expression from any of the promoters tested (data not shown). This suggests that repressive effects of the HRT N-terminal region require DNA-binding domains.

HRT mRNA Accumulates in Various Tissues—Northern blotting with the full-length HRTd cDNA probe was used to examine the pattern of accumulation of HRT mRNA in barley tissues (Fig. 7). The same blots were also probed for comparison with an Amy1 cDNA (pMC; Ref. 24) and with a gene-specific fragment of the cDNA encoding GAMyb, a presumptive, GA-dependent activator of a-amylase transcription (19). Amy1 and GAMyb mRNAs were also detected in immature seed tissues, whereas Amy1 mRNA was not (Fig. 7, lanes 8–10). Neither HRT nor Amy1 mRNAs were detected in dormant, dry seed...
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The term “zinc finger” was introduced to describe the conserved C-terminal zinc finger domains in many plant proteins (20). These domains consist of two or more highly conserved Cys-His pairs that are coordinated by a central zinc ion, allowing the fingers to bind to target sequences separated by spacers of various lengths, thereby increasing specificity (47).

The accumulation of the three mRNAs was also examined in embryos and aleurone layers from early harvested, immature seeds and from mature, germinating seeds cultured for 24 h in the absence or presence of exogenous GA (Fig. 7, lanes 14–19). These incubations did not produce detectable Amy1 mRNA in tissues from immature seeds. This was expected, as Amy1 is not normally expressed in immature or dormant seeds (20). However, both HRT and GAMyb mRNAs did accumulate in the cultured, immature tissues (Fig. 7, lanes 8–10 versus lanes 14–17). The same treatments of aleurone layers isolated from mature, germinating seeds showed that Amy1 mRNA levels increased severalfold during incubation with GA (Fig. 7, lanes 18 and 19). GAMyb mRNA levels were also high following either control or GA treatment. In contrast, HRT mRNA levels were barely detectable in layers from germinating seeds.

DISCUSSION

We previously delineated promoter regions that mediate hormone-responsive expression of α-amylase genes in barley (GARE and GARC; Refs. 12, 13, and 15). These sequences were used as probes in southwestern screens to isolate HRT. Data base searches with HRT sequences revealed homology only to HRTdb1–3) represent a novel C3H zinc finger motif in which three conserved cysteines and a histidine function as ligands for a central Zn2+ ion resulting in a domain capable of binding DNA (Figs. 2 and 3). Four cysteine/histidine residues are also conserved in the zinc fingers of TFIII A from Xenopus (44), although with a different consensus sequence. The term zinc finger was introduced to describe the motifs of TFIII A but is now used to describe a diverse set of protein motifs capable of binding Zn2+ (45). Many zinc finger proteins are of the cluster type containing multiple tandemly repeated fingers separated by a conserved short sequence. However, several zinc finger proteins, in which the fingers are separated by much longer spacers of various lengths, have been described from plants (46). The spacing between the three fingers in HRT is also wide. In the cluster type proteins the fingers bind contiguous triplet sequences in the target DNA. The wider spacing between the fingers in the plant proteins may introduce structural flexibility and may allow the fingers to bind to target sequences separated by spacers of various lengths, thereby increasing specificity (47).

The repressive effects of HRT on transcription in plant cells from the CaMV 35 S and maize Ubi promoters (Fig. 5) indicates that HRT may repress transcription from promoters which lack a canonical GARE. This suggests that endogenous HRT may repress the expression of numerous genes including α-amylases. Sequence comparison of the promoters of Amy1/6–4, Amy2/32b, CaMV 35 S, and maize Ubi1 identified the following similar sequences: Amy1/6–4, –148 GCCGATAACAACTCT; Amy2/32b, –130 TCTC-GTAACAGA-GTC; CaMV 35 S, –503 GGAC-CTAACAGACTC; maize Ubi1, –312 GGCGTAAACAGG-CTG (bottom strand). Further analyses are required to determine whether HRT binds these or other sequences in the CAMV 35 S and maize Ubi1 promoters.

Evidence presented here indicates that α-amylase is a target for HRT repressor activity. First, HRT preferentially binds GARC and GARE sequences in vitro (Fig. 2). Second, full-length HRT and truncated forms of HRT affected expression from α-amylase promoters. Thus, HRT repressed GA-repressed transcription (Fig. 5, A and B), and HRTΔ2+NLS increased the level of transcription in the absence of hormone from both the Amy1/6–4 and Amy2/32b promoters (Fig. 6, A and B). In contrast, when an ABRE was substituted for the GARE in the Amy2/32b promoter, full-length HRT (Fig. 5E) and HRTΔ2+NLS (not shown) had no significant effect on ABA-dependent transcription from this chimeric promoter. Third, Northern blotting showed that HRT mRNA accumulates in tissues where Amy1 mRNA does not accumulate (Fig. 7). Although these inversely correlated patterns of mRNA accumulation may be fortuitous, they are in keeping with a role for HRT in regulating α-amylase gene expression.

Further modeling of mechanisms by which HRT may regulate the transcription of genes such as α-amylase requires consideration of other putative regulatory factors. Gubler et al. (19) presented evidence that GAMyb specifically binds to an Amy1 GARE, and that GAMyb activates expression from an Amy1 promoter in cells not treated with GA to the same level as that in cells treated with the hormone. They also showed that GAMyb mRNA accumulates earlier following GA treatment than Amy1 mRNA, and that GAMyb mRNA accumulation is induced by cycloheximide. This suggests that α-amylase transcription is dependent upon accumulation of GAMyb whose expression is derepressed by GA. Correlative evidence for a repressor function may be seen in the absence of Amy1 mRNA.

FIG. 7. HRT mRNA accumulates in various tissues. Three Northern blots of total cellular RNA (20 µg) from barley tissues (top) were probed sequentially with radiolabeled cDNAs encoding HRT, Amy1, or a wheat ribosomal cDNA (left). These RNAs were from vegetative tissues of 40-day-old greenhouse plants (lanes 1–7); whole seeds and dissected embryo and distal, half-seed starchy endosperm and aleurone harvested while immature at 25 days postanthesis (lanes 8–10), or when fully mature at 45 days (lanes 11–13); embryos and aleurone layers isolated from the same lots of immature (lanes 14–17) or mature, germinating seeds (lanes 18 and 19) incubated with or without 10 µM GA. Blots probed with HRT and GAMyb were exposed 9 days, with Amy1 1 day, and RNA for 6 h.
in immature, cultured tissues which accumulate high levels of HRT and GAMyb (Fig. 7). This suggests that either Amy1 expression is not solely dependent upon GAMyb, or that HRT, or another factor, is capable of repressing Amy1 expression in the presence of GAMyb.

Studies in other systems indicate that GA signaling involves a repressor function(s). In Arabidopsis, the SPY tetratricopeptide repeat protein and the putative transcription factors GAI and RGA are involved, directly or indirectly, in pathway(s) that negatively regulate GA responses (4, 6, 7). In maize, the transcription factor VP1 mediates repression of α-amylase expression solely during seed development (20). This developmental role and the fact that α-amylase induction in germinating maize is largely independent of GA (20) make it difficult to ascertain a function for a VP1 homologue in the germinating barley aleurone system used here to study HRT. Future biochemical studies on barley HRT and molecular genetic analyses of the Arabidopsis HRT homologue may be pursued to clarify the roles of these proteins in plants.

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