EXPRESSION PATTERNS OF *ARABIDOPSIS DRG* GENES: PROMOTER-GUS FUSIONS, QUANTITATIVE REAL-TIME PCR, AND PATTERNS OF PROTEIN ACCUMULATION IN RESPONSE TO ENVIRONMENTAL STRESSES

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DRGs are very highly conserved GTP-binding proteins. All eukaryotes contain DRG1 and DRG2 orthologs. *Arabidopsis* has three DRGs: *AtDRG1* (At4g39520), *AtDRG2* (At1g17470), and *AtDRG3* (At1g72660). DRG2 and DRG3 encode proteins that are 95% identical; identity between DRG1 and DRG2/3 is 55%. The focus of this article is expression of *Arabidopsis* DRGs. DRG1 and DRG2 promoter-GUS constructs showed similar spatial expression in seedlings and mature organs, but gene-specific differences were noted. Quantitative real-time PCR experiments indicated similar levels of DRG1 and DRG2 mRNA accumulation in most tissues. DRG3 transcripts were very low in all tissues. Heat stress at 37°C led to a 10-fold increase in DRG1 transcripts and a 1000-fold increase in DRG3 transcripts. DRG1 antibodies recognized a 43-kD protein, and DRG2 antibodies recognized bands at 30, 43, and 45 kD. Plants were exposed to stresses (salt, heat, cold, UV light, osmotic, and other stresses) and examined by Western blotting. Only heat stress caused detectable changes. Heat did not affect DRG1, but DRG2 and a 72-kD protein recognized by DRG2 antibodies both increased. The modest changes in DRG mRNA and protein levels seen here suggest that other types of regulation, such as altered subcellular localization, may be important for their cellular functions.

**Keywords:** DRG, GTPase, GTP-binding protein, heat stress, environmental stress.

Introduction

GTP-binding proteins (G proteins) regulate a wide variety of cellular activities in all organisms (Bourne et al. 1990, 1991; Bischoff et al. 1999; Leipe et al. 2002). Some well-characterized G proteins are Go of heterotrimeric G proteins, small monomeric G proteins (including Ras, Ran, Rab, Rho, and Arf), several translation initiation and elongation factors (bacterial IF2, EF-G, EF-Tu, and their eukaryotic counterparts), and components of signal-recognition particles and their receptors. Heterotrimeric G proteins are widely used in eukaryotic signal transduction pathways, including those in plants (Jones and Assmann 2004). Ras, which is critical for regulating cell proliferation in animals, is one of the few G proteins that does not occur in plants. ROPs, which are related to Rho G proteins, may carry out Ras-like activities in plants (Yang 2002; Vernoud et al. 2003). Rab proteins provide specificity for vesicle targeting, Rho contributes to organization of the actin cytoskeleton, Ran regulates transport into and out of nuclei, and Arf is involved in vesicle assembly (Bischoff et al. 1999). The functions of many other subfamilies of G proteins remain poorly understood.

The availability of complete genome sequences from a large number of bacteria, archaea, and eukaryotes has led to a better understanding of the origins and evolutionary relationships of GTPases. A thorough phylogenetic analysis was based on the structure of the GTP-binding pocket and of various effector domains (Leipe et al. 2002). The origins of ~10 major G-protein families can be traced to the last universal common ancestor of all living organisms, suggesting that they perform essential physiological activities. Among these 10 families are four translation-factor families, two additional families of predicted translation factors, two signal-recognition particle-associated GTPases, and two OBG-like GTPases, OBG and DRG. OBG is an essential gene in *Bacillus subtilis* (Moriyama et al. 2002). Genes for nuclear-encoded OBGs in eukaryotes are presumed to have originated in the bacterial ancestors of mitochondria and chloroplasts. DRGs, the focus of this report, occur in archaea and eukaryotes. All 10 ancient families of G proteins associate with ribonucleoprotein complexes and probably play important roles in RNA metabolism in extant cells (Caldon et al. 2001; Leipe et al. 2002).

DRGs from archaea and unicellular eukaryotes are assigned to COG 1163 (http://www.ncbi.nlm.nih.gov/COG/). Each archaean species has a single DRG gene, whereas eukaryotes contain members of two orthologous groups, DRG1 (KOG 1487) and DRG2 (KOG 1486). All eukaryotes appear to contain at least one representative of each group (Li and Trueb 2000). A mouse *DRG* was the first to be characterized; it is the archetype of the DRG1 group (Sazuka et al. 1992a, 1992b). A human DRG became the archetype of the DRG2 group (Schenker et al. 1994). Both DRG1 and DRG2 from most organisms contain ~365–370 amino acid residues and have molecular masses of ~43 kDa. Amino acid identity of DRGs from plants, animals, and fungi is very high: identity...
within an orthologous group is ~65%–70%, whereas paralogs from a single species share ~55%–60% identity. The guanine nucleotide-binding pocket (G1–G5 motifs) of DRG1 and DRG2 proteins is contained roughly between residues 60 and 290. The C-terminus contains a TGS domain (pam02824), which also occurs in threonyl-tRNA synthase and E. coli SpoT, and is suggested to be an RNA-binding domain (Wolf et al. 1999).

In Arabidopsis, the only DRG to be characterized to date is encoded by At1g17470. We previously referred to this protein as AtDRG (Devitt et al. 1999), whereas another group called it AtDRG1 (Etheridge et al. 1999). Based on a more recent naming scheme (Li and Trueb 2000), this gene and its encoded protein will be referred to as AtDRG2 and AtDRG2 (or simply DRG2), respectively. Arabidopsis contains two additional DRG genes. The sequence of At4g39520 shows clear affinity with the DRG1 orthologous group, so it will be called AtDRG1. We refer to the last gene, At1g72660, as AtDRG3. AtDRG2 and AtDRG3 encode proteins containing 399 amino acids that are 95% identical to each other.

Some aspects of DRG mRNA and protein accumulation have been studied in several organisms. Two studies on animal DRGs are of note because they examined the expression of both DRG1 and DRG2 in their respective systems. Li and Trueb (2000) studied steady state levels of both mRNAs by Northern blotting of mouse and human tissues. In mouse, both mRNAs were moderately abundant in testis, kidney, liver, brain, and heart and were reduced or absent in skeletal muscle, lung, and spleen. Both messages were present in 12 human tissues but varied somewhat in abundance. In all cases, though, the relative levels of DRG1 and DRG2 mRNAs were similar. InSV40-transformed fibroblasts, DRG2 levels were low and DRG1 levels were high, suggesting a compensatory interaction between the expression of these genes (Li and Trueb 2000; see also Schenker et al. 1994). Ishikawa and co-workers (2003) studied the expression of Xenopus DRG1 and DRG2 by Northern blotting and in situ hybridization. Both mRNAs accumulated steadily and rather similarly during Xenopus development. The exception to this generality was seen at the earliest stages of development, where XDRG1 was absent and XDRG2 was moderately abundant. In adult tissues, both messages were moderately abundant in most tissues (XDRG1 was present at very low levels in heart, lung, and liver) and highly abundant in testis and ovaries. In situ hybridization revealed similar but not identical spatial patterns of expression of these two genes.

Using Northern blots, we previously showed that pea DRG2 mRNA accumulates preferentially in tissues that are in a growing state (Devitt et al. 1999). We also showed that Arabidopsis DRG2 expression parallels that of a histone gene, which is a marker for actively dividing and elongating cells (Devitt et al. 1999). We concluded that plant DRG2 genes are broadly expressed but that there is a somewhat greater level of expression in some growing, dividing, or metabolically active cells and tissues. In yeast two-hybrid assays, human DRG1 was found to interact with the TAL1/SCL proto-oncoprotein, suggesting a role in cell-cycle control (Mahajan et al. 1996; Zhao and Aplan 1998). Immunolocalization studies of Arabidopsis DRG2 protein (which the authors called AtDRG1) showed that this protein occurs in punctate granules, but the identity of these granules or organelles remains unknown (Etheridge et al. 1999).

In this article, I focus on expression patterns of Arabidopsis DRG genes using promoter-GUS fusions involving the DRG1 and DRG2 promoter regions (called prDRG1 and prDRG2), transcript accumulation of all three genes using quantitative real-time PCR (qRT-PCR), and protein accumulation on Western blots using DRG1- and DRG2-specific antibodies.

Material and Methods

Plants

Arabidopsis thaliana, ecotype Wassilewskija (Ws), was used for all experiments. Roots (Rt) and young leaves (YL) were from 2-wk-old plants grown sterilely on vertically oriented MS plates at 25°C under continuous light. For all other tissues, plants were grown in ProMix in a growth chamber at 20°C.

To examine the effects of photoperiod, plants first were grown for 25 d under short-day photoperiods (SD; 10L:14D). Some plants then were transferred to long-day photoperiods (LD; 16L:8D). SD and LD samples were collected after a total of 28 d. All other tissues were collected from SD plants, including old rosette leaves (OL) from 6-wk-old plants, inflorescence stems (St), flower buds (Bd), open flowers (Fl), and green silique (Si). To test for response to various environmental stresses, seeds were sown on horizontal MS agar plates, cold treated at 4°C in the dark for 3 d, and then grown for 9–10 d in a growth chamber at 25°C in continuous light. Plants then were exposed to a particular agent for 6 or 24 h. For chemical treatments, 85-mm round plates were flooded with 10 mL of solution. Plants were kept in the growth chamber during the treatment periods, except for temperature treatments at 4°C or 37°C, which were carried out in a dark refrigerator or incubator, respectively. Both dark and light controls were performed. The treatments tested were UV light (a single exposure of 105 μJ delivered using a Stratagene Stratalinker); 275 mM mannitol; 25 μM etidium bromide; 100 mM NaCl; 100 μM glyphosate; 1 mM NaAsO2 (arsenite); 1 mM Na2HAsO4 (arsenate); and 100 mM K-phosphate at pH 4.4, 5.6, 7.4, and 8.8. Tissues for qRT-PCR and Western blots were frozen in liquid nitrogen and stored at −80°C.

Cloning, Sequencing, and Construction of GUS Constructs

Work on the DRG2 promoter predated the availability of Arabidopsis genomic sequence. A previously described DRG2 cDNA clone (Devitt et al. 1999) was used to screen the CD4–8 genomic library in the A-Fix vector (obtained from the Arabidopsis Biological Resource Center [ABRC]). We isolated and fully sequenced a clone containing an insert of ~5 kb, which included ~1.6 kb of DNA upstream of the ATG start codon. Four regions of the DRG2 promoter region were amplified by PCR and cloned into the Agrobacterium binary vector pBI101. Sequences of the PCR primers were F1 (within λ–Fix), GTTCCTGAGCCGACGCGATATACCC; F2, TAGTGGACGTTTAATTCTAGAG; R1, CGGCGATCCCGCGAAGAGAGG; and R2, CGGCGATCCTCACCTGTCTTCGCTAC (restriction sites added for cloning are underlined). The following primer pairs were used.
to produce four DRG2 promoter constructs: construct I, F1 and R2; construct II, F2 and R2; construct III, F1 and R1; and construct IV, F2 and R1 (see fig. 1). These clones were transformed into Agrobacterium strain GV3101, and the resulting cells were used to transform Ws Arabidopsis plants by vacuum infiltration (Bechtold and Pelletier 1998). Kanamycin-resistant plants were selected on MS plates, tested for the presence of the appropriate DNA by PCR, and brought to homozygosity.

The DRG1 promoter region was cloned based on the published genomic sequence. Sequences of the forward and reverse primers were TGTGTGACTGGTTAATATCGAGAGCTGAAGG and ACTCCATGGTTGCAGTCAAAGCACAG, respectively. These primers amplified a 628-bp fragment, which was cloned into binary vector pCAMBIA 1382. This clone was transformed into Agrobacterium strain GV3101, which was used to transform Ws Arabidopsis plants by the floral dip method (Clough and Bent 1998). Hygromycin-resistant plants were selected on MS plates, tested for the presence of the appropriate DNA by PCR, and brought to homozygosity.

Histochemical detection of GUS activity was carried out by incubating plants or plant parts for 4–18 h in 0.5 mM X-gluc (5-bromo-4-chloro-3-indolyl-b-D-glucopyranoside) in a buffer containing 50 mM Na-phosphate, pH 7.0, and 0.1% Triton X-100 (Jefferson et al. 1987; Beeckman and Engler 1994). Chlorophyll was cleared through several changes of 70% ethanol. Digital images were captured directly with a camera or with a light microscope using brightfield or darkfield optics.

**Antisera and Western Blotting**

DRG2 antibodies (antiserum no. 55) were generated by immunizing rabbits with a His-tagged fusion protein containing the N-terminal 202 residues of pea DRG2 (Devitt et al. 1999). The entire 369-residue coding region of AtDRG1 was cloned into pQE30 (Qiagen) in order to generate a fusion protein containing a His-tag at its N-terminus. Forward and reverse PCR primers corresponding to the AtDRG1 cDNA were used to amplify the entire coding region (AGAGGATCC-ATGTCGACTATTATGCAAGAGATGGACTCATATCITTTTTACGATCTGAACACAC, respectively). A cDNA clone was used as a template (clone M70001 in pBlueScript SK− from ABRC). Rabbits were immunized by injecting antigen into surgically implanted ball chambers (Clemons et al. 1992). DRG1 antibodies (antiserum no. 29) were affinity purified using an AffiGel-10 column (BioRad) to which DRG1-His had been covalently bound.

Proteins were extracted by grinding in extraction buffer on ice (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 29 mM b-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using a dye-binding assay (BioRad). Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% or 12% acrylamide gels using standard techniques. In a given experiment, either 50 or 100 μg of protein was loaded in each lane. Equal loadings were verified by staining identical gels with Coomassie brilliant blue (these control gels are not shown). Proteins were

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**Fig. 1** Structure of Arabidopsis DRG1, DRG2, and DRG3 genes. DRG1 encodes a predicted protein containing 369 residues with a mass of 41.1 kDa. DRG2 and DRG3 encode proteins containing 399 amino acids with masses of ~44.6 and 44.8 kDa, respectively. The sizes and positions of coding exons of DRG2 and DRG3 are identical (coding exons are shown in black and noncoding exons are shown in gray). Exon 1 and intron 1 of DRG2 and DRG3 are within the 5′ noncoding regions of these genes. The “up” and “down” arrows indicate the limits of the coding regions of upstream and downstream genes (arrowheads show the positions of stop codons). Upstream promoter regions of DRG1 and DRG2 (Prom) were fused to β-glucuronidase (GUS) to make transcriptional fusions. One promoter construct was generated for DRG1, and four constructs were made for DRG2.
electrophoretically transferred to nitrocellulose or polyvinylidene difluoride membrane blots using a semidry apparatus. Blots were incubated overnight in Tris-buffered saline (TBS; 20 mM Tris, pH 7.5, 500 mM NaCl) with affinity-purified primary antibodies at dilutions ranging from 1:100 to 1:1000. Following washes in TBST (TBS plus 0.05% Tween-20), blots were incubated for 1–2 h with HRP-DAR secondary antibodies in TBS at a dilution of 1:5000 (donkey-antirabbit antibodies conjugated to horseradish peroxidase, Amersham). A second series of washes in TBST ensued. Finally, blots were incubated in SuperSignal West Pico chemiluminescence substrate (Pierce) and exposed to x-ray film.

Quantitative Real-Time PCR

Total cellular RNA was purified using the RNAqueous-4PCR kit together with Plant RNA Isolation Aid (both from Ambion). Residual genomic DNA was hydrolyzed by DNAase treatment. Reverse transcription was carried out using the RETROscript kit (Ambion). The resulting cDNAs were used as templates for qRT-PCR.

In most cases, each primer spanned two exons, and there was at least one additional exon within the amplicon. Structure of three DRG genes is summarized in figure 1. The coding sequences of DRG2 and DRG3 are very similar, so forward primers for these genes were based on 5′ noncoding sequences (in each of these genes, exon 1 and intron 1 are wholly contained within 5′ noncoding DNA). The sequences of these primers were DRG1qF (spans exons 2 and 3), CTTTCTCATCTGGTGTATAGCGGG; DRG1qR (spans exons 5 and 6), TGATGTCCT-TAGCCGTACTTAAACCTGGTCTTCCTACCTTTTC; DRG2qF (spans exons 1 and 2), GGAGATCGCCCTCTACACATTTCCACTATAGTAGCAAAAACAGG; DRG2qR (spans exons 4 and 5), GACTTTCGCCACACTGAAATCTCATAAGTGCAACAGGTC; DRG3qF (spans exons 1 and 2), CGG-TAGACAATCTGTGCACAGGGAATGGTGAAGATACCTAGCTC; and DRG3qR (within exon 4), CCCATACTTTGTAACCTCAAAACACTCCACCACCTCCACTAGT. The actin-8 gene (At1g49240) was used as an internal control. Primers for this gene were Actin8F, TCACGACCTTTCAACGAGATT, and Actin8R, ATGCCTGACCTGCTCAT. For end-point PCR, DRG gene-specific primers were used to amplify cDNA templates for 40 amplification cycles at an annealing temperature of 65°C. The products were electrophoresed on agarose gels and visualized by ethidium bromide fluorescence. To verify the identity of these PCR products, bands were isolated from the gels, cloned into pGEM-T-Easy (Promega), and sequenced.

Quantitative RT-PCR reactions were carried out in an Mx3000P real-time PCR system (Stratagene). A master mix was prepared using 1.25 μL each of forward and reverse primers, each at 10 ng/μL, 6.75 μL water, and 0.25 μL of a 100× dilution of reference dye R4526 (Sigma). For each reaction, 9.5 μL of the mastermix, 3 μL of an appropriate template (water or cDNA), and 12.5 μL of SYBR Green JumpStart Taq ReadyMix (Sigma S4438) were mixed in a final volume of 25 μL. Negative controls included no-template control (water) for each primer pair to measure interference due to primer-dimer formation and a DNAase-treated RNA control to assess contamination from genomic DNA. The standard cycling conditions were as follows: denaturation (94°C for 2 min) followed by 40 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 30 s) and final extension (72°C for 1 min). Data were collected at the end of each annealing step. The cycle threshold (Ct) for each sample was generated by MxPro software. The Ct value for each sample corresponded to the point at which the fluorescence crossed the threshold. Fluorescence from SYBR Green increases as double-stranded DNA accumulates (Morrison et al. 1998). Following amplification, characterization of products was performed by melting-curve analysis (95°C for 1 min, 55°C for 30 s, 95°C for 30 s). Fluorescence data were continuously collected as the temperature ramped up from 55°C to 95°C. The dissociation curve for each sample was generated by MxPro software to determine the melting temperature ($T_m$) of the reaction product or products using the value = -ΔRn (T), which is the first derivative of the normalized fluorescence reading multiplied by −1. Relative expression data presented here are normalized to 10,000 actin transcripts. Replicate experiments showed similar results.

Results

Structure of Arabidopsis DRG Genes

The Arabidopsis genome contains three DRG genes, which we refer to as DRG1 (At4g39520), DRG2 (At1g17470), and DRG3 (At1g72660). The positions and sizes of introns, exons, 5’ and 3’ untranslated regions (UTRs), ORF limits of upstream and downstream genes, and other features are shown in figure 1. DRG1 encodes a protein of 369 amino acid residues. DRG2 and DRG3 encode proteins containing 399 residues. Overall amino acid identity between DRG1 and DRG2/DRG3 is ~56%, whereas identity between DRG2 and DRG3 is 95%. Canonical GTP-binding domains include five G boxes and two switch regions. These motifs all occur between residues 80 and 290 of each DRG protein. A TGS domain, which may be involved in RNA binding, spans residues 290–365. DRG proteins from nearly all organisms contain ~365–370 residues. A 32-amino-acid “tail” at the C-terminus of Arabidopsis DRG2 and DRG3 also occurs in pea DRG2 (GenBank AF014821). The coding region of DRG1 contains 10 exons. DRG2 and DRG3 each contain 12 exons. Exon 1 and intron 1 of these two genes are wholly contained within their 5′ UTRs.

Promoter Constructs and Expression

Upstream promoter regions of DRG1 and DRG2 were fused with β-glucuronidase (GUS) to make transcriptional fusions. Several attempts also were made to generate transgenic plants expressing the DRG3 promoter fused with GUS. Although hygromycin-resistant plants were isolated, none of these showed reliable GUS expression in any tissue or in response to heat stress. The λ CD4–8 genomic library was screened using a DRG2 cDNA as a probe. The largest clone isolated was ~5.0 kb, including ~1.6 kb of DNA upstream of the ATG start codon. This clone was fully sequenced (J. P. Stafstrom, unpublished data). Exon 1 of DRG2 was 74 bp in length. Four promoter constructs were generated by PCR and then cloned into pBI101. These constructs contained either 982 or 448 bp of DNA upstream of exon 1, either with or without intron 1 (427 bp; fig. 1). Transgenic plants were generated that contained each promoter construct. GUS expression
Fig. 2  Expression in *Arabidopsis* tissues of DRG1 and DRG2 promoter-GUS transcriptional fusions. GUS activity was assayed in transgenic plants containing fusions to *prDRG1* (a, c, e, g, i, k, m, o, q) or *prDRG2* (b, d, f, h, j, l, n, p, r). Tissues analyzed were 7-d-old seedlings (a, b),
was not detected from promoter constructs that lacked the intron (constructs III and IV; data not shown). Expression from constructs I and II was strong in many tissues, and spatial and temporal expression patterns appeared to be identical (data not shown). It is now apparent that construct II begins near the stop codon of the upstream gene and that construct I extends into that gene (fig. 1). All data shown below are from the longest construct, construct I. A single prDRG1-GUS construct was generated in pCAMBIA 1382 based on the Arabidopsis genomic sequence. This promoter region contained 628 bp of DNA immediately upstream of the ATG start codon.

Expression of prDRG1-GUS and prDRG2-GUS was examined in cells and tissues throughout the plant life cycle. In general, both promoters were active at many stages of development. In seedlings, prDRG1 was strongly expressed in cotyledons, hypocotyls, and roots, particularly at the root apex and in lateral root primordia (fig. 2a). prDRG2 was expressed in the blades of cotyledons and was strongly expressed in roots, but it was not expressed in hypocotyls or the petioles of cotyledons (fig. 2b). Rosette leaves of plants grown in short photoperiods expressed both genes (fig. 2c, 2d). However, prDRG1 was more strongly expressed in vascular tissues and prDRG2 was quite specifically expressed in trichomes (fig. 2c, 2d, insets). Inflorescences contain flowers at progressive stages of development. prDRG1 was expressed predominantly in anthers and stigmas (fig. 2e). Much or all of the “anther staining” is due to staining of pollen within these anthers (pollen staining is seen more clearly in fig. 2i). prDRG2 also was expressed strongly in anthers and stigmas and, in addition, it was expressed in sepals and petals (fig. 2f). Both genes were expressed in the receptacle regions of older flowers, particularly in abscission scars of sepals, petals, and stamens (fig. 2g, 2h). prDRG2 was expressed in siliques walls (fig. 2i). A closer examination of roots using prDRG1 antibodies showed that GUS staining was more strongly expressed in vascular tissues and prDRG1 was expressed predominantly in roots and stems containing relatively low levels and flower buds, open flowers, and green siliques containing relatively high levels. DRG3 transcripts could be detected in most tissues, but their levels were ∼30-fold to 100-fold lower than those of DRG1 or DRG2.

**Western Blotting**

Patterns of DRG1 and DRG2 protein accumulation were analyzed on Western blots using specific, affinity-purified antibodies. DRG2 antibodies recognized protein bands with apparent molecular masses of 45, 43, and 30 kDa (fig. 5). The amount of each band was variable in different tissues. The smaller bands are proteolytic products of the 45-kDa band (B. Nelson, K. Maas, J.-M. Dekeyser, and J. Stafstrom, unpublished manuscript). In control experiments, DRG2 antibodies barely recognized DRG1-His (not shown). DRG1 antibodies are highly specific for DRG1, which has an apparent molecular mass of 43 kDa. Portions of the same batches of tissues that were analyzed by qRT-PCR (fig. 4) were used to prepare

6-wk-old rosettes grown in short photoperiods (c, d; insets show details of single leaves), inflorescences (e, f), receptacle regions following organ abscission (g, h), siliques wall and stigma (i, j), shoot apex region of young seedlings (k, l), and seedling roots at various stages of lateral root development (m–r). Identified structures or organs are anther (An), hypocotyl (Hy), lateral root (LR), pollen (P), petal (Pe), shoot apex (SA), sepal (Se), stipule (Stp), stigma (St), and trichome (T).

**Quantitative Real-Time PCR**

Gene-specific primers were designed to carry out qRT-PCR for each DRG gene (see “Material and Methods” for details). Several precautions were taken to ensure that quantitative data were due to amplification of a specific gene and of cDNA derived from mRNA, not genomic DNA. Total RNA, which was used to produce cDNA by reverse transcription, was treated with DNAase to destroy genomic DNA. Also, primers were designed to include at least one exon within the amplicon that was not complementary to the primers. Complementary DNAs prepared from several tissues were subjected to non-quantitative end-point PCR (fig. 3). In every case, only a single band was amplified by each pair of primers. The size of each PCR product was identical to the size that would be amplified from cDNA. Finally, an example of each band was subcloned into pGEM-T-Easy and sequenced. Each sequence exactly matched that of the expected cDNA (data not shown).

Complementary DNAs isolated from several tissues were used as templates for quantitative RT-PCR (fig. 4). Melting-curve analysis of each transcript indicated the presence of a single species, which is further evidence for the specificity of these reactions (data not shown). Replicate experiments gave the same results. Transcript numbers were normalized to actin-8 transcripts (Ct values relative to 10,000 actin transcripts). In each tissue, the levels of DRG1 and DRG2 transcripts were similar. A comparison between tissues indicated a range of ∼10-fold in the levels of each of these two transcripts, with roots and stems containing relatively low levels and flower buds, open flowers, and green siliques containing relatively high levels. DRG3 transcripts could be detected in most tissues, but their levels were ∼30-fold to 100-fold lower than those of DRG1 or DRG2.
protein samples for Western blot analysis (fig. 5). The levels of DRG1 in each tissue were quite similar, although noticeably lower levels were present in siliques and leaves of SD and LD plants. DRG2 levels were considerably more variable. The 45-kDa band was highly abundant only in flower buds, whereas the 43-kDa band was present in roots, stems, young leaves, flower buds, and open flowers. Low levels of the 30-kDa band were present in stems, flower buds, and open flowers. Very little (if any) of the three DRG2 bands was detected in old leaves, leaves of SD or LD plants, or siliques.

Accumulation of DRG proteins in response to a variety of chemicals and to alterations of the physical environment were examined (fig. 6). Samples were collected from seedlings grown on agar plates after 6 or 24 h of treatment. The 30-kDa DRG2 band was not present in any sample, so this region of the blots is not shown. Since some treatments occurred in the dark and others in the light, controls under both conditions were performed. The presence or absence of light did not affect accumulation of either protein (fig. 6a). In fact, none of the treatments tested had a large effect on the accumulation of the 43-kDa DRG1 band or the 43/45-kDa DRG2 bands. These treatments included exposure to 4°C, UV light, mannitol, ethidium bromide, NaCl, glyphosate, Na-arsenite, Na-ar senite, or pH over the range of 4.4–8.8. An exception was the response of DRG2 to heat stress at 37°C. Heat stress led to an increase of the 43-kDa band at 6 h, and it also led to the appearance of a 72-kDa protein that was recognized by DRG2 antibodies (fig. 6a). A time-course experiment showed that increased accumulation of the 45- and 72-kDa bands begins 3 h after the onset of heat stress (fig. 6b). These bands persisted at elevated levels through 24 h of continuous heat stress. During recovery from heat stress (6 h at 37°C, followed by a return to 25°C), the 72-kDa band was still present after 6 h (6 + 6 sample) but was gone after 24 h (6 + 24 sample).

**DRG mRNA Expression in Response to Heat Stress**

Accumulation of DRG transcripts in response to heat stress was examined by qRT-PCR (fig. 7). During exposure to heat stress at 37°C, DRG2 transcript levels declined about eightfold over 24 h. In contrast, DRG1 transcripts increased about eightfold during the first 3 h of heat stress and then declined gradually to their initial levels by 24 h. DRG3 transcripts, which were barely detectable in tissues grown under normal conditions, increased more than 1000-fold within 3 h of the onset of heat stress. Accumulation of DRG transcripts during recovery from heat stress was also examined (fig. 7). Plants first were exposed to 37°C for 3 h and then allowed to recover at 25°C. Following 3 h of recovery (3 + 3 samples), DRG2 transcript levels were unchanged (cf. the 3-h sample), DRG1 transcripts declined slightly, and DRG3 transcript levels declined ~10-fold. At later recovery stages (3 + 9 and 3 + 21 samples), the level of each transcript changed little from the 3 + 3 stage. Heat treatments occurred in a dark incubator. The 0 + 24 sample was a control that was kept in the dark but was not subjected to heat stress.

**Discussion**

Very little is known about the cellular functions of DRG1 or DRG2 from any organism. It is also unknown whether they perform similar functions. This is quite surprising given the very high level of sequence conservation among DRGs and the many important functions performed by other types of G proteins. An understanding of where and when these genes are involved is necessary to understand their roles in plant development.

![Fig. 3](image-url) **Fig. 3** End-point PCR using qRT-PCR primers using gene-specific PCR primers. Complementary DNAs prepared from young leaves (YL), leaves of 4-wk-old plants grown under short (SD) or long (LD) photoperiods, flower buds (Bd), open flowers (Fl), and green siliques (Si) were used as templates for standard PCR reactions. Water was used as a negative control. An agarose gel stained with ethidium bromide is shown. Each pair of primers produced a single band of the expected size. Sequencing one band of each type confirmed its identity.

![Fig. 4](image-url) **Fig. 4** qRT-PCR analysis of DRG transcripts from Arabidopsis tissues. Complementary DNAs were prepared from the same six tissues shown in fig. 3 as well as from roots (Rt), stems (St), and old leaves (OL). The actin-8 gene was used as an internal control for quantitative comparisons. Relative expression values based on measured cycle threshold values were normalized to 10,000 actin transcripts. In each of these tissues, there were similar levels of DRG1 and DRG2 transcripts. Between tissues, the transcript levels varied by up to 10-fold (e.g., roots vs. flower buds). DRG3 mRNA was present at very low but detectable levels in most tissues.
expressed and how this expression is controlled by internal and external factors could reveal aspects of their function. Also, such information would certainly provide a vital foundation for ongoing biochemical, cellular, and genetic studies.

In this study, expression patterns of *Arabidopsis* DRG genes were examined using three approaches. Each approach can provide useful information, but each has its limitations as well. First, qRT-PCR was used to determine steady state levels of DRG mRNAs (figs. 4, 7). Using several types of controls, we established that the Ct threshold values reported were specific to a particular gene. This consideration was especially important for DRG2 and DRG3, in which the nucleotide sequences of their coding regions were 90% identical. Similar results to those reported here are available from microarray databases. Nevertheless, it is important for individual investigators to repeat these experiments, especially when, for example, relative cellular levels of mRNAs and proteins do not correspond with one another. Second, Western blotting was used to assess DRG1 and DRG2 protein levels. DRG1 antibodies were highly specific for a 43-kDa band and never recognized a 45-kDa band, which is the size of the largest form of DRG2. We have established through other work that the 43-, 45-, and 30-kDa bands recognized by DRG2 antibodies are breakdown products of DRG2 (B. Nelson, K. Maas, J.-M. Dekeyser, and J. Stafstrom, unpublished manuscript). DRG2 antibodies recognize DRG1-His only very weakly, so the 43-kDa band recognized by these antibodies is predominantly DRG2. Our third approach was to generate transgenic plants expressing promoter-GUS transcriptional fusions. This was a very useful adjunct to the other approaches because neither of these could reveal cellular patterns of gene expression. Our focus was on the promoters of DRG1 (prDRG1) and DRG2 (prDRG2), because we were unable to obtain verifiable transgenic plants containing prDRG3 fused to GUS. Transgenic plants were generated that contained four different DRG2 promoter constructs fused to GUS (fig. 1). Constructs III and IV lacked intron 1, which is wholly contained within the 5′ UTR. GUS expression was not seen in plants containing these constructs, so cis elements within the intron might be important for high levels of promoter activity. Intron-mediated enhancement of gene expression has been documented for a number of other genes (Callis et al. 1987; Mascarenhas et al. 1990; Rose 2004).

Steady state levels of DRG1 and DRG2 mRNAs were similar to each other in all of the tissues that were tested. Also, there was only ~10-fold difference between the tissues with the highest and lowest levels of accumulation of each mRNA (fig. 4). Based on the tissues analyzed, these genes are broadly expressed, a general conclusion that corroborates that of microarray experiments (Craigon et al. 2004; Zimmermann et al. 2004; Schmid et al. 2005). Aliquots of the same tissue samples were used for qRT-PCR (fig. 4) and for Western blotting (fig. 5). The levels of DRG1 protein were similar in these tissues. In contrast, DRG2 levels were highly variable; significant amounts of the 45-kDa complete protein were found only in flower buds, and very little of any of the three forms of DRG2 could be detected in old leaves, plants grown under SD or LD photoperiods, or siliques. Because the DRG2 mRNA levels in these tissues were very similar (fig. 4), the discrepancy between protein and mRNA accumulation in siliques, old leaves, and leaves of SD and LD plants may be due to differential protein synthesis, degradation, or both within these tissues. We previously noted discrepancies in the relative levels of DRG2 mRNA and protein in pea axillary buds (Devitt et al. 1999). In this case, however, protein levels were constant, whereas mRNA levels were more abundant in growing buds than in dormant buds. DRG1 and DRG2 from *Xenopus*, human, and mouse are susceptible to polyubiquitination and degradation by the 26S proteosome pathway (Ishikawa et al. 2005). Each DRG protein is stabilized through an interaction with a specific DRG family regulatory protein (DFRP). It is hypothesized that this interaction prevents polyubiquitination. *Arabidopsis* contains DFRP homologues, but it is not known whether they play a similar role in stabilizing DRGs.

The very high level of sequence conservation among DRGs from eukaryotes and archaea and the quite similar bacterial OBGs suggest that they play an important and perhaps related role in all of these organisms. The broadly similar patterns of mRNA accumulation that we have observed indicate that transcription is not the primary level at which the DRGs are regulated. Still, accumulation of DRG proteins might vary in
response to some internal or external stimulus, such as a hormone or an environmental stress. We tested a variety of stresses and other treatments using young plantlets grown on MS plates. Experimental parameters (e.g., concentrations, durations of treatments, etc.) were selected based on the *Arabidopsis* Gantlet Project (http://thale.biol.wwu.edu/) and other sources. The conditions we used were generally similar to those published recently by the Harter Laboratory (Kilian et al. 2007). The conditions tested were high and low temperatures, genotoxic stress (UV light and ethidium bromide), osmotic stress (mannitol), salinity, amino acid starvation (glyphosate), arsenate (heavy metal toxicity), arsenite (oxidative stress), and pH over the range of 4.4–8.8 (fig. 6). Most of these treatments had little or no effect on the accumulation of *DRG1* or *DRG2*. Heat stress led to increases in the *DRG2* 45-kDa band and to a 72-kDa band that was recognized by *DRG2* antibodies. The 72-kDa protein appeared within 3 h of the onset of heat stress and persisted through 24 h. This protein was still present following 6 h of recovery at 25°C but was absent after 24 h of recovery. 

![Graph showing protein accumulation patterns](image)

**Fig. 6** Patterns of *DRG* protein accumulation in response to chemical and environmental stresses. Plants were grown on MS plates for 9 d. Some treatments were in the light and others were in the dark, so controls for both are included. *a–c,* Plants were exposed to the following conditions and collected 6 or 24 h after the onset of the treatment (treatment was continuous except for UV light): 37°C, 4°C, UV light (single exposure to 10⁵ μJ), 275 mM mannitol, 25 μM ethidium bromide, 100 mM NaCl, 100 μM glyphosate, 1 mM Na-arsenite, 1 mM Na-arsenate, and 100 mM K-phosphate at pH 4.4, 5.6, 7.4, or 8.8. The effect of heat stress at 37°C also was analyzed over a time course of 0.25–24 h. Recovery from heat stress at 37°C for 6 h was analyzed after an additional 6 or 24 h at 25°C. Most treatments had little effect on the accumulation of *DRG1* or *DRG2*. Heat stress led to increases in the *DRG2* 45-kDa band and to a 72-kDa band that was recognized by *DRG2* antibodies. The 72-kDa protein appeared within 3 h of the onset of heat stress and persisted through 24 h. This protein was still present following 6 h of recovery at 25°C but was absent after 24 h of recovery. 

![Graph showing protein accumulation patterns](image)
protein appeared as early as 3 h after the onset of heat stress and persisted at high levels for the duration of this stress (Kilian et al. 2007). Given that DRG1 and DRG2 are broadly expressed and that their encoded proteins are quite similar, single mutants might be complemented during normal development, partially or fully, by the other gene. Since DRG3 is hardly expressed except in response to heat stress, a mutation in this gene might reveal a phenotype only under stress conditions. Although prDRG1 and prDRG2 showed overlapping patterns of activity, some differences were seen: prDRG1 was more abundantly expressed in hypocotyls, petioles of cotyledons, and vascular tissue of old leaves, and prDRG2 was preferentially expressed in sepals, petals, silique walls, and trichomes. We previously found that pea and Arabidopsis DRG2 transcripts accumulated preferentially in tissues and organs defined to be in a growing state. Here we see that the DRG1 and DRG2 promoters are very active in root apices at several stages of development (fig. 2m–2r). However, neither promoter was active in the shoot apex region of seedlings (fig. 2k, 2l).

There are many further avenues to pursue regarding DRG function. Although steady state levels of DRG1 and DRG2 proteins change little in response to most stress conditions (fig. 6), the activity of these proteins could be regulated by posttranslational modifications, subcellular localization, altered rates of synthesis and/or degradation, or other means. Arabidopsis DRG2 occurs in punctate organelles or granules (Etheridge et al. 1999), but the identity of these granules in not known, nor are the conditions that might cause DRGs to associate with them. Although DRGs contain all of the sequence motifs that are hallmarks for GTP binding and hydrolysis, GTP binding has been demonstrated experimentally only for DRG1 from mouse and Drosophila (Sazuka et al. 1992; Sommer et al. 1994). GTP-binding properties of plant DRG1 proteins have not been documented, nor have the binding properties of DRG2 from any organism. OBGs, bacterial G proteins that are closely related to DRGs, provide many useful paradigms for analyzing DRG function. For example, OBGs are essential for cell viability, are involved in stress signaling, and physically interact with ribosomes (Kobayashi et al. 2001; Buglino et al. 2002; Lin et al. 2004; Czyz and Wegrzyn 2005; Foti et al. 2005). DRG2 (or DRG3) appears to be involved in heat stress responses. We are testing whether DRGs interact with ribosomes, which might then alter translation in some manner. Xenopus DRGs interact with DFRPs (Ishikawa et al. 2005). Arabidopsis contains apparent homologues of these DFRPs, so we are interested in knowing whether and how these other proteins interact with plant DRGs.

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**Literature Cited**


