

# Cosuppression of Eukaryotic Release Factor 1-1 in Arabidopsis Affects Cell Elongation and Radial Cell Division

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The role of the eukaryotic release factor 1 (eRF1) in translation termination has previously been established in yeast; however, only limited characterization has been performed on any plant homologs. Here, we demonstrate that cosuppression of *eRF1-1* in Arabidopsis (*Arabidopsis thaliana*) has a profound effect on plant morphology, resulting in what we term the *broomhead* phenotype. These plants primarily exhibit a reduction in internode elongation causing the formation of a broomhead-like cluster of malformed siliques at the top of the inflorescence stem. Histological analysis of *broomhead* stems revealed that cells are reduced in height and display ectopic lignification of the phloem cap cells, some phloem sieve cells, and regions of the fascicular cambium, as well as enhanced lignification of the interfascicular fibers. We also show that cell division in the fascicular cambial regions is altered, with the majority of vascular bundles containing cambial cells that are disorganized and possess enlarged nuclei. This is the first attempt at functional characterization of a release factor in vivo in plants and demonstrates the importance of eRF1-1 function in Arabidopsis.

Protein synthesis is an essential process for all living organisms. Translation of mRNA into protein basically consists of three stages: (1) initiation, involving the assembly of the ribosomal subunits at the 5' end of an mRNA, (2) elongation, the process of tRNA-mediated decoding of the mRNA to form a polypeptide chain, and (3) termination, during which a stop codon signals the end of translation, and the ribosomal subunits dissociate from the mRNA. Each stage requires specific accessory proteins or factors.

The signal that indicates the end of a polypeptide is the presence of an in-frame stop codon (UAA, UGA, or UAG) at the ribosomal A-site. In eukaryotes, termination of protein synthesis is carried out by two classes of release factors: eukaryotic release factor 1 (eRF1) and eRF3 (Frolova et al., 1994; Kisselev and Frolova, 1995; Stansfield et al., 1995; Zhouravleva et al., 1995; Frolova et al., 1996). The stop codon is recognized by eRF1, which binds the ribosome and promotes hydrolysis of the ester bond linking the polypeptide chain with the tRNA in the ribosomal P-site (Tate and Caskey, 1974; Caskey, 1980; Frolova et al., 1994). Because eRF1 has a number of structural and functional similarities to a tRNA molecule, it has been hypothesized to enter the ribosomal A-site and catalyze the termination reaction through tRNA mimicry (Íto et al., 1996; Song et al., 2000). Interaction between eRF1 and eRF3 C-terminal regions results in a conformational rearrangement of

either eRF1 or both factors in the complex (Frolova et al., 1998). The function of eRF3 is to stimulate the activity of eRF1 through the hydrolysis of GTP (Zhouravleva et al., 1995), although the presence of eRF3 in human cells has been shown to be nonessential for termination (Le Goff et al., 1997).

Homologs of eRF1 have been identified in a wide range of eukaryotes, including human, frog (*Xenopus laevis*), nematode (*Caenorhabditis elegans*), ciliate (*Tetrahymena thermophila*), and fungus (*Podospora anserina*). Despite considerable differences between these organisms, a high degree of sequence similarity exists among homologs. Complementation studies have shown that this sequence similarity also translates into functional similarity. *Xenopus eRF1* cDNA expressed in a yeast (*Saccharomyces cerevisiae*) mutant carrying a temperature-sensitive *eRF1* allele, *eRF1* (ts), rescued the conditional-lethal phenotype of the mutant at 36°C (Tassan et al., 1993). Other studies using human, rabbit, and Syrian hamster eRF1 homologs have also been shown to complement *eRF1* (ts) in yeast (Urbero et al., 1997; Karamyshev et al., 1999).

To date, three *eRF1* genes (*eRF1-1*, *eRF1-2*, and *eRF1-3*) have been identified in Arabidopsis (*Arabidopsis thaliana*) by sequence homology (Brown et al., 1995), and all three genes have been shown to possess release factor activity (Chapman and Brown, 2004). In this article, we describe an unusual phenotype resulting from cosuppression of *eRF1-1* in Arabidopsis. Cosuppressed plants exhibit a reduction in internode elongation and are affected in radial cell division. Based on these findings, we discuss the implications of down-regulating a release factor and explore the possibility of an alternative function for *eRF1-1*.

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## RESULTS

### The Broomhead Phenotype

Homozygous *Arabidopsis* transgenic lines were produced that overexpress an endogenous *eRF1-1* cDNA (GenBank accession no. H37361). The *eRF1-1* overexpression lines predominantly produced phenotypically normal plants, and we refer to these transgenics as nonphenotype plants. However, a limited percentage of plants (3%–60%) across multiple transgenic lines displayed an abnormal *broomhead* phenotype (Fig. 1A). The most distinctive feature of the phenotype is the reduction in internode spacing of the inflorescence stems, creating a broomhead-like structure. Compared to wild-type plants, *broomhead* plants are reduced in height (Table I), produce malformed siliques, and synthesize anthocyanin in nearly all mature plant tissues (Fig. 1, B and C). No significant difference in time to flowering was observed between *broomhead* and wild-type plants (Table I). However, *broomhead* plants flowered for longer, and there was an increase in the total number of inflorescence meristems formed and a reduction in number of seeds produced per pod (Table I). During juvenile rosette development, wild-type and *broomhead* plants are identical in appearance (Fig. 1, D and E). The onset of the *broomhead* phenotype primarily occurs during reproductive development, although the more severe transgenics exhibit signs of the phenotype in the adult vegetative stage through the production of anthocyanin in the veins of the rosette leaves (Fig. 1, F and G). In less severe transgenics, the onset of the phenotype is preceded by the production of anthocyanin in the inflorescence stem below the bud. As the phenotype progresses, the synthesis of anthocyanin subsequently occurs in the rosette and cauline leaves.

Typically, only a small percentage of plants in a specific line show the *broomhead* phenotype, and not all of these plants display the phenotype to the same degree. Differences in plant height, number of inflorescences produced, total seed production, and the height of the primary bolt can be observed within a single homozygous line (Fig. 2, A–D). The *broomhead* structures also vary in the degree of clustering, and sometimes *broomhead* plants revert to producing wild-type inflorescences (Fig. 2, E–H). Plants displaying the most severe phenotype terminate growth of the primary inflorescence at the rosette level. A decrease in phenotype severity is correlated with an increase in the height of the primary inflorescence and the corresponding production of *broomhead* structures further away from the rosette.

### The Broomhead Phenotype Is Caused by *eRF1-1* Cosuppression

Expression levels of the endogenous *eRF1-1* gene as well as the transgene were determined by northern analysis performed on a variety of tissues from both *broomhead* and wild-type plants. In most tissues exam-



**Figure 1.** Wild-type and *broomhead* phenotypes. A, A mature wild-type plant (left) and a *broomhead* plant exhibiting a severe phenotype (right). B, Wild-type inflorescence showing regular spacing of flowers along the internode. C, Reduced internode elongation in the *broomhead* phenotype. Wild-type (D) and *broomhead* (E) plants are identical during juvenile rosette development. However, at the late adult vegetative stage, *broomhead* transgenics (G) are distinguishable from wild-type plants (F) by the production of anthocyanin in the veins of the rosette leaves.

ined, *eRF1-1* transcript levels were significantly higher in the transgenics than in the wild type (Fig. 3A). However, in the stem and bud tissue where the *broomhead* phenotype is most visible, significant degradation of the *eRF1-1* transcript was observed. To exclude the possibility of poor RNA quality, the northern was re-probed with a second unrelated gene, *AtDRG2*, encoding a cytoplasmic G-protein (At4g39520). Although some degradation was observed in all *broomhead* samples, most of the *AtDRG2* transcripts remained intact (Fig. 3A). Probing with a ribosomal gene also failed to reveal any significant degradation products. This suggests that the degradation of *eRF1-1* in the stem and bud tissues of *broomhead* plants is transcript specific.

**Table 1.** Developmental characteristics of wild-type and broomhead plants

Parameter	Wild Type <sup>a</sup>	Broomhead <sup>a</sup>
Plant height (cm)	37.1 ± 1.8	18.5 ± 6.5
Days to flowering	27.9 ± 1.3	28.3 ± 1.9
Days to termination of flowering	47.1 ± 1.2	64.5 ± 2.0
Total number of inflorescence meristems	5.3 ± 1.1	15.8 ± 5.2
Number of seeds per pod	60.8 ± 6.3	34.0 ± 23.3

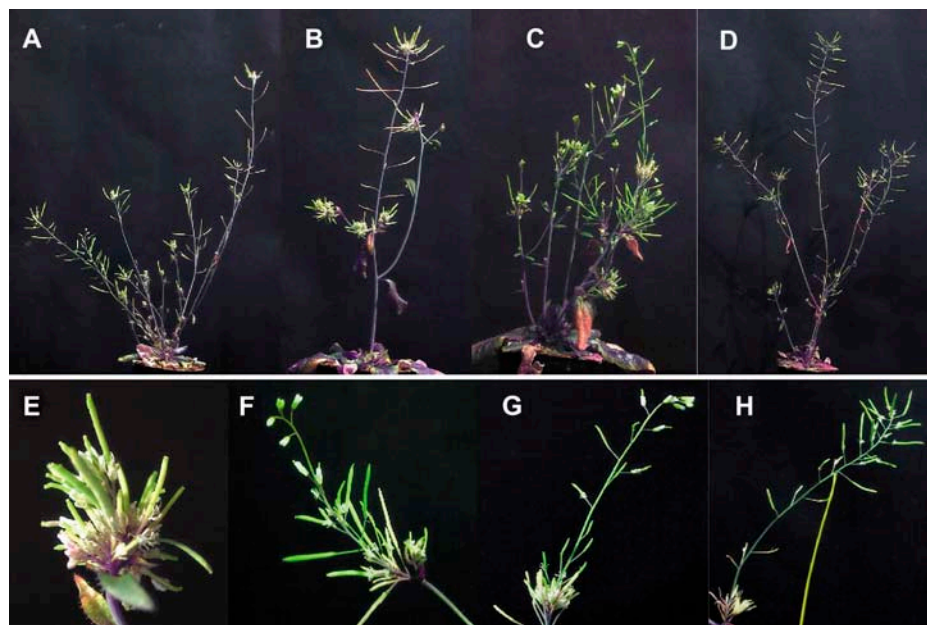
<sup>a</sup>Results shown are the average ± SD taken from 17 wild-type and 19 broomhead plants.

The possibility that the phenotype was due to an insertional effect was ruled out by southern analysis on four independent broomhead lines that clearly shows different chromosomal integration sites for each line (Fig. 4). The degradation observed in broomhead plants was specific to plants showing the phenotype as *eRF1-1* transcript levels were up-regulated in nonphenotype transgenic plants from six different lines with no significant degradation products observed (Fig. 3B).

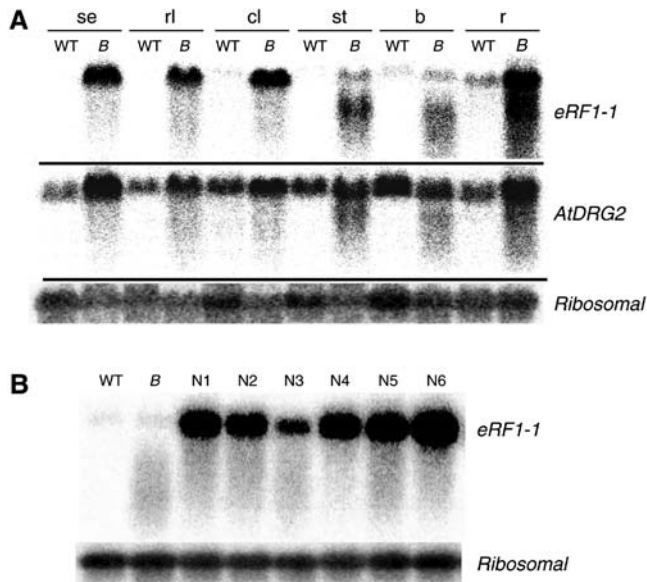
To investigate the link between transgene degradation and the broomhead phenotype, an *eRF1-1*: $\beta$ -glucuronidase (*GUS*) in-frame gene fusion was introduced into Arabidopsis plants under the control of the 35S promoter of cauliflower mosaic virus (CaMV 35S). A number of lines carrying this hybrid construct showed the characteristic broomhead phenotype, and homozygous lines were selected and analyzed in further detail. All severe broomhead phenotype transgenics failed to show *GUS* activity anywhere in the plant (Fig. 5A). These plants did not bolt above the rosette level, and no viable seed was produced. However, broomhead plants from less severe lines showed *GUS* activity in the developing seeds (Fig. 5B). This is in agreement with other cosuppression reports where

gene silencing is reset during seed development (Hart et al., 1992; Deborne et al., 1994; Kunz et al., 1996; 2001). Staining of the nonphenotype plants was less consistent; approximately half of the plants exhibited extensive *GUS* staining (Fig. 5C), while the other half remained largely unstained (Fig. 5D). *GUS* activity in the mostly unstained plants was only apparent in the older rosette leaves either at the tips or in the area surrounding the veins (Fig. 5E) and in developing seeds (Fig. 5F). This *GUS* staining pattern is suggestive of a downward systemic silencing signal, as has been reported previously during cosuppression of *GFP* in tobacco (*Nicotiana tabacum*; Voinnet et al., 1998).

Northern analysis confirmed the presence of the *eRF1-1*:*GUS* transcript in the blue nonphenotype plants (Fig. 5G, top panel, lane 3). In contrast, only a faint signal was observed for the transgene in broomhead transgenic bud tissue (Fig. 5G, top panel, lane 5), and no transcript was detected in *GUS* negative nonphenotype bud tissue (Fig. 5G, top panel, lane 4). When the endogenous levels of *eRF1-1* were detected using the 3' untranslated region of the gene (absent in the *eRF1-1*:*GUS* transgene) as a probe, only the broomhead tissue displayed down-regulation of the transcript (Fig. 5G, middle panel). This suggests that posttranscriptional silencing of both the transgene and endogenous gene occurs in broomhead plants, even though down-regulation of *eRF1-1* is not complete. The presence of a single band followed by a smear in the blue nonphenotype and broomhead RNA samples (Fig. 5G, top panel, lanes 3 and 5) may be indicative of a cosuppression event, although the blue nonphenotype samples still exhibit high levels of the transgene and wild-type levels of the endogenous *eRF1-1* gene. It is possible that cosuppression has been triggered in these plants but has not yet had the chance to progress very far. The absence of the *eRF1-1*:*GUS* transgene in *GUS* negative nonphenotype



**Figure 2.** Variation in the broomhead phenotype at the whole plant level and in the broomhead-like structures. A to D, Broomhead plants showing growth retardation of the primary bolt at the rosette level and the production of numerous auxiliary bolts (A), prominent broomhead-like structures on the inflorescence apices (B), an intermediate severity phenotype and the presence of multiple auxiliary bolts (C), and a weak phenotype with broomhead-like structures appearing later in development (D). E to H, Inflorescence stems showing a severe reduction in internode elongation (E) and a reversion of the phenotype with newly formed inflorescences exhibiting an increase in internode elongation (F–H).



**Figure 3.** Northern analysis of wild-type, *broomhead*, and nonphenotype tissues. A, Northern blot of total RNA prepared from different tissues (*se*, seedlings; *rl*, rosette leaves; *cl*, cauline leaves; *st*, stem; *b*, buds; *r*, roots) of wild-type (WT) and *broomhead* (*B*) plants. The membrane was hybridized with a radiolabeled *eRF1-1* probe and subsequently stripped and hybridized with an *AtDRG2* probe. B, Total RNA was isolated from bud tissue of nonphenotype transgenics (N1–N6) in six independent *eRF1-1* overexpressor lines. Wild-type and *broomhead* RNA bud samples were included as controls. A ribosomal probe for both blots was used to verify equal sample loading.

plants is also consistent with a silencing effect, although it appears that only the transgene is silenced in these plants, as the endogenous *eRF1-1* transcript levels were unaffected.

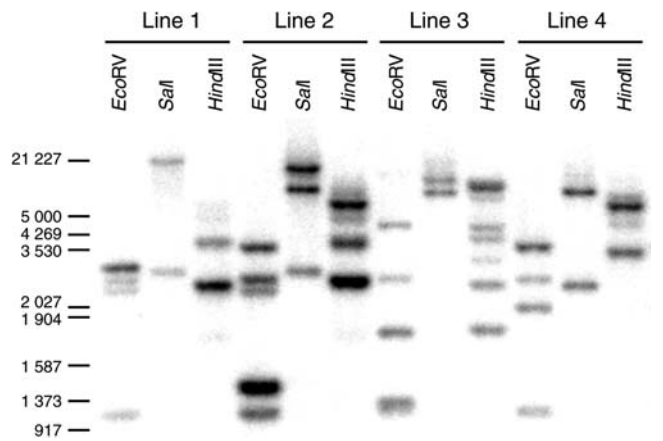
***eRF1-1* Family Member Transcripts Are Down-Regulated in *Broomhead* Tissue**

*eRF1-1* is part of a gene family that includes two other members, *eRF1-2* (At1g12920) and *eRF1-3* (At3g26618). At the nucleotide level, these family members show 76% (*eRF1-2*) and 77% (*eRF1-3*) identity to *eRF1-1* and 87% identity to each other. To determine if either the *eRF1-2* or *eRF1-3* endogenous gene is affected in *broomhead* phenotype plants, quantitative real-time PCR was performed on bud tissue with specific primers for each family member and compared to wild-type controls. In wild-type and nonphenotype transgenics, *eRF1-2* and *eRF1-3* transcript levels were almost identical (Fig. 6A, white bars). However, in *broomhead* transgenics, *eRF1-2* and *eRF1-3* transcript levels were down-regulated to approximately half of the wild-type levels (Fig. 6A, black bars). When total *eRF1-1* (endogenous gene and transgene) transcript levels were quantified, an average of 7-fold and 3.8-fold overexpression was detected in the nonphenotype and *broomhead* transgenics, respectively (Fig. 6B). This shows down-regulation of *eRF1-1* in

*broomhead* plants relative to nonphenotype plants, although not below wild-type levels. Down-regulation of *eRF1-2* and *eRF1-3* indicates that the silencing effect is not restricted to *eRF1-1* but also affects the family members, most likely due to their high sequence homology.

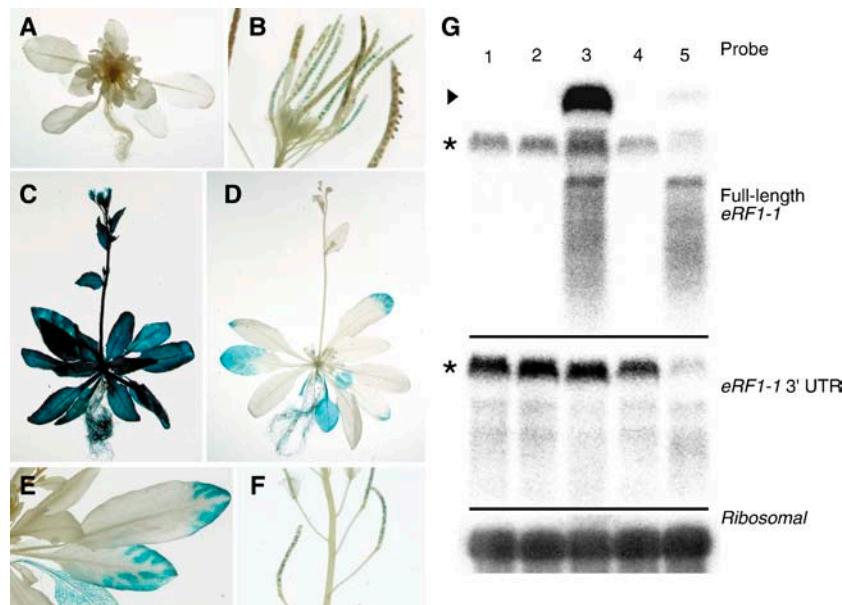
**Cellular Architecture of the *Broomhead* Phenotype**

To study the *broomhead* phenotype at the anatomical level, histological analyses were performed on longitudinal and transverse sections of the *broomhead* inflorescence stem and the corresponding region in wild-type plants. Microscopic examination revealed that a reduction in cell elongation rather than cell division was responsible for the decrease in plant height. In general, the average pith cell length was reduced approximately 10-fold in *broomhead* inflorescence stems (17.33 μm) relative to the wild type (174.5 μm; Fig. 7, A and B), and the cells were more cytoplasmically dense. No morphological aberrations were observed in the apical meristem, suggesting that cell division in this region is unaffected (Fig. 7, D and E). However, the majority of cambial cells were disorganized and hypertrophic and contained enlarged nuclei (Fig. 7C). The nuclei were visualized with safranin-O, which stains chromosomes and nucleoli in addition to lignified tissue. Further analysis of transverse stem sections (Fig. 7, F–J) revealed that most fascicular cambial regions exhibit this disorganized cell growth, although a few fascicular cambial regions appeared more active and contained an increased number of cell layers (Fig. 7I). Several layers of interfascicular fiber precursor cells were also identified in *broomhead* stems that were absent in the wild-type stems (Fig. 7, F and G). In addition, ectopic lignification of the phloem cap cells and some phloem sieve cells as well as enhanced lignification of the interfascicular fibers were



**Figure 4.** Southern-blot analysis of four independent *broomhead* lines. Genomic DNA was extracted from *broomhead* lines and digested with *EcoRI*, *Sall*, or *HindIII*, transferred to a membrane, and probed with a radiolabeled *eRF1-1* probe.





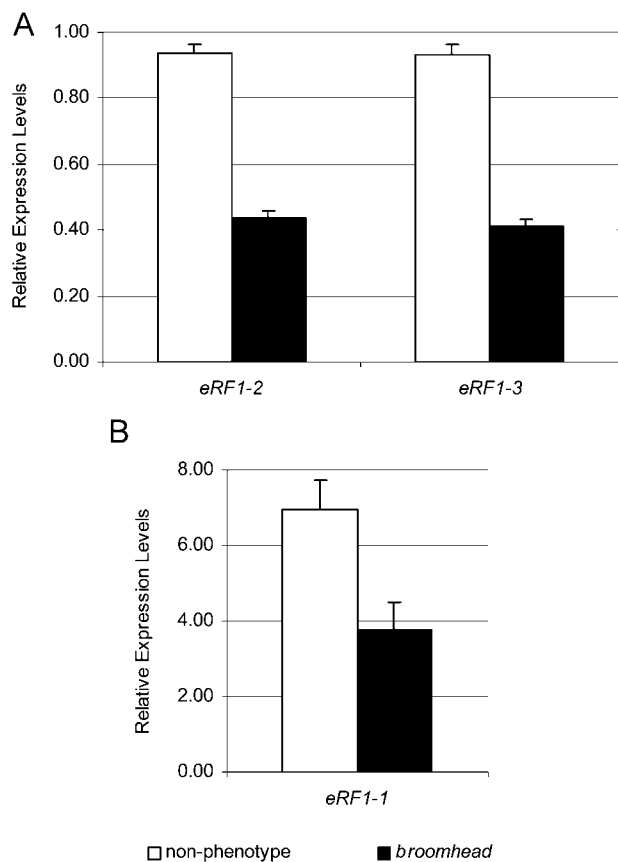
**Figure 5.** GUS staining pattern and northern analysis of *eRF1-1:GUS* transgenic *Arabidopsis* plants. A, An *eRF1-1:GUS* transgenic plant exhibiting a severe *broomhead* phenotype and failing to show any GUS activity. B, A *broomhead* cluster from a less severe transgenic line revealing GUS activity in developing seeds. C and D, Nonphenotypic transgenics showing GUS activity in all plant tissues (C) and minimal GUS activity in rosette leaves (D). E, Close-up of GUS staining pattern in rosette leaves. F, A mostly GUS negative nonphenotypic plant exhibiting GUS activity in developing seeds. G, Northern analysis of total RNA isolated from bud tissue of wild-type plants (lane 1), CaMV 35S-*GUS* (transgenic control, lane 2), and *eRF1-1:GUS* transgenics (lanes 3, 4, and 5). *eRF1-1:GUS* transgenics were grouped according to their GUS expression pattern and phenotype: GUS positive nonphenotypic plants (lane 3), GUS negative nonphenotypic plants (lane 4), and GUS negative *broomhead* plants (lane 5). The cauline leaves of nonphenotypic transgenics were stained at the time of bud collection to ensure the separation into GUS positive and negative samples. The membrane was hybridized with a probe corresponding to the full-length *eRF1-1* transcript to allow detection of both the *eRF1-1:GUS* transgene and the endogenous *eRF1-1* gene. The membrane was then stripped and rehybridized to the 3' untranslated region (UTR) of the *eRF1-1* transcript to only enable detection of the endogenous gene. Hybridization with a ribosomal probe was used to show equal loading in each lane. An arrowhead indicates the *eRF1-1:GUS* transcript and the endogenous *eRF1-1* is indicated by an asterisk.

observed in *broomhead* stems (Fig. 7, G, I, and J). Some areas of the fascicular cambium that had become disorganized also showed evidence of lignification (Fig. 7, G and J). To confirm that the safranin-O stain was in fact detecting the presence of ectopic lignin, wild-type and *broomhead* stem sections were treated with the lignin-specific stain phloroglucinol-HCl (Fig. 7, K and L). As expected, *broomhead* stem sections stained pink/red around the walls of the phloem cap cells and phloem sieve cells and also in some regions of the disorganized fascicular cambium (Fig. 7L), indicating the presence of lignin. Compared to wild-type sections, enhanced lignification was also observed in the interfascicular fibers. These results confirm the presence of enhanced and ectopically lignified cells in *broomhead* plants, as observed with the safranin-O stain.

#### *eRF1-1* Expression Is Localized to the Vascular Tissues and Actively Growing Regions

To examine the endogenous expression pattern of *eRF1-1*, approximately 2 kb of the *eRF1-1* promoter was fused to the *GUS* reporter gene, and a number of *Arabidopsis* transgenic lines carrying the construct

were analyzed. Under normal growth conditions, *GUS* expression was detected at all developmental stages, including germination and seedling development, and in mature plants (Fig. 8, A–F). The most obvious expression was seen in the vascular tissues at all growth stages, particularly in the phloem and also in the cambium, xylem parenchyma, and interfascicular parenchyma cells (Fig. 8, G and H). However, in the elongating stem region just below the buds, a more uniform pattern of expression was observed across all cell types (Fig. 8I). Other areas of *GUS* positive staining included pollen grains, trichomes, and guard cells (Fig. 8, J–L). Younger and more actively growing tissues, i.e. germinating seeds, the shoot apical meristem, and root meristem, exhibited the strongest *GUS* staining (Fig. 8, A, B, and F). These results are consistent with the expression pattern observed in the northern analysis of wild-type plants (Fig. 8M), where high levels of the *eRF1-1* transcript were seen in closed and open buds and in the roots. The basal level of *eRF1-1* expression in the rosette and cauline leaves also correlates with the low level of *GUS* activity detected in the leaf veins. Promoter-*GUS* fusions of the two family members (*eRF1-2* and *eRF1-3*) were also



**Figure 6.** Relative expression levels of *eRF1-1*, *eRF1-2*, and *eRF1-3* family members in nonphenotype and *broomhead* transgenic plants. *eRF1-1*, *eRF1-2*, and *eRF1-3* transcript levels were quantified in wild-type, *broomhead*, and nonphenotype plants using quantitative real-time PCR. The graphs show the relative values, with respect to wild-type plants, of *eRF1-2* and *eRF1-3* (A) and *eRF1-1* (B) in *broomhead* plants (black bars) and nonphenotype plants (white bars). Values shown are means  $\pm$  SE.

constructed, and *Arabidopsis* transgenic lines were produced and characterized. The patterns of *GUS* expression found in these lines were identical to the ones observed for *eRF1-1* (data not shown), indicating that there is no complementarity in expression patterns between family members and suggesting a possible redundancy in function.

## DISCUSSION

### The *Broomhead* Phenotype Is a Result of Gene Silencing

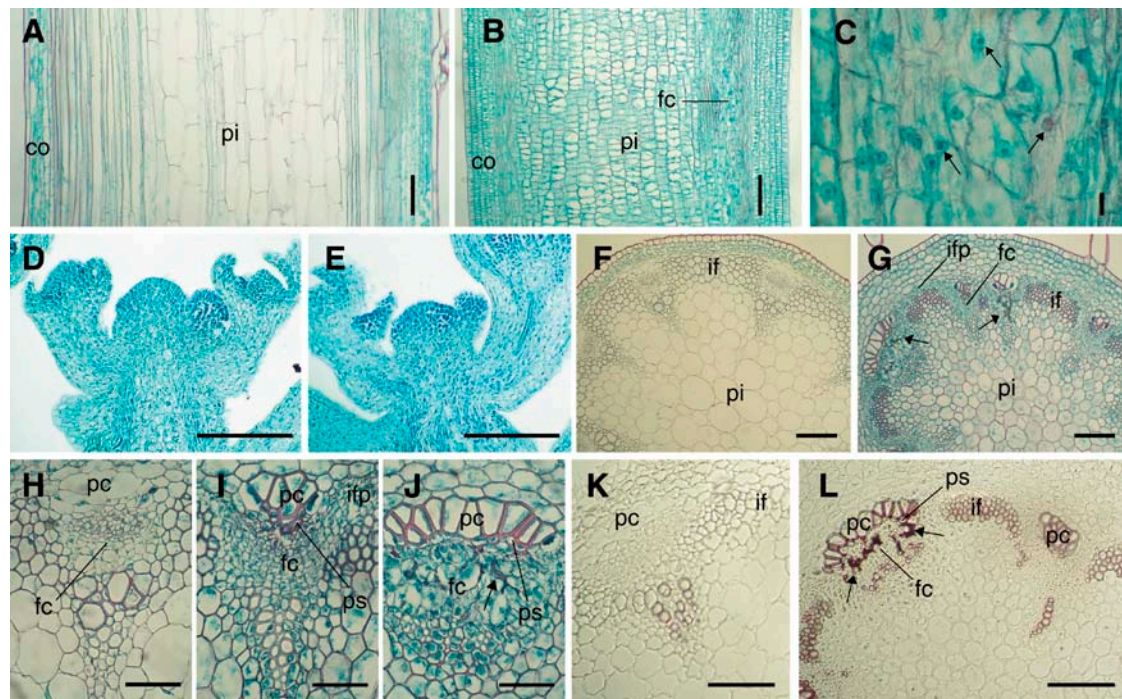
Our evidence indicates that the *broomhead* phenotype in *Arabidopsis* lines engineered to overexpress *eRF1-1* is a result of gene silencing. First, northern analysis on *broomhead* plants revealed that *eRF1-1* transcripts were partially degraded in tissues showing the phenotype. In contrast, those plants not exhibiting a phenotype displayed overexpression of the gene with no significant degradation products. A similar degradation smear below the full-length transcript on a northern has been reported for transgenic *N. ben-*

*thamiana* plants in which the *LUCIFERASE* gene had been silenced (Kjemtrup et al., 1998). In addition, the occurrence of truncated, polyadenylated, and non-polyadenylated RNAs corresponding to 5' and 3' regions of posttranscriptionally silenced genes has been previously described (Smith et al., 1990; Goodwin et al., 1996; Lee et al., 1997; Metzloff et al., 1997; Tanzer et al., 1997; Han and Grierson, 2002), suggesting that transgene degradation is gene specific.

Second, all *broomhead* plants containing the 35S-*eRF1-1*:*GUS* gene fusion failed to show *GUS* activity in any plant tissues, except for the developing seeds (Fig. 5A). This indicates that at least the transgene is being silenced in *broomhead* phenotype plants, and the *GUS* activity in the developing seeds represents the post-meiotic resetting of the silencing event and has been described in a number of reports (Hart et al., 1992; Deborne et al., 1994; Kunz et al., 1996, 2001). Northern analysis revealed that in addition to the transgene being down-regulated in *broomhead* plants, the endogenous *eRF1-1* transcript was also down-regulated relative to wild-type plants (Fig. 5G). Taken together, the reduction in transgene and endogenous *eRF1-1* transcript levels provides strong evidence for cosuppression.

Third, quantitative real-time results revealed that the transcript levels for family members *eRF1-2* and *eRF1-3* were also reduced relative to nonphenotype and wild-type plants. It has been suggested that at least 60% to 70% identity in the coding sequence is the threshold necessary to produce posttranscriptional gene silencing of homologous genes (Kunz et al., 2001). As both family members satisfy this requirement with 76% and 77% identity to *eRF1-1*, it is likely that *eRF1-2* and *eRF1-3* are also targets of posttranscriptional gene silencing in *broomhead* plants. Whether down-regulation of all three family members is needed to produce the *broomhead* phenotype is not yet clear, although considering the functional redundancy among family members, it seems likely that all three *eRF1* genes would need to be silenced in order to produce the *broomhead* phenotype.

The failure to detect overall lower levels of the *eRF1-1* transcript in 35S-*eRF1-1* *broomhead* transgenics does not necessarily imply that all the *eRF1-1* transcripts are functional and able to be translated. In fact, one of the models proposed to explain how posttranscriptional gene silencing is induced is via the production of aberrant RNA from the homologous transgene. The aberrant RNA is suggested to act as a template for the degradation of itself and other homologous RNAs. Because we were unable to distinguish between the endogenous *eRF1-1* transcript and the transgene, due to their similarity in length and sequence, we could not confirm whether or not the endogenous gene was down-regulated. However, based on the findings that (1) a significant portion of the *eRF1-1* transcript is already partially degraded in *broomhead* plants, (2) both family members are down-regulated, and (3) 35S-*eRF1-1*:*GUS* *broomhead* transgenics show down-regulation of



**Figure 7.** Anatomy of inflorescence stems and apical meristems of wild-type and *broomhead* plants. A and B, Longitudinal sections of the mid-region of wild-type (A) and *broomhead* (B) inflorescence stems. C, Enlargement of the fascicular cambial region in B showing disorganization of the cambium. Enlarged nuclei are marked by arrows. D and E, Longitudinal sections of wild-type (D) and *broomhead* (E) apical meristems. F and G, Transverse sections of the mid-region of wild-type (F) and *broomhead* (G) stems. The arrows indicate lignification of the disorganized fascicular cambial region. H and I, Enlargement of the vascular bundles from F and G. H, Wild type; I and J, *broomhead* vascular bundles. The fascicular cambial region displays active, controlled cell division in I and disorganized cell growth with enlarged nuclei in J. The arrow in J indicates lignification of the disorganized fascicular cambial region. K and L, Phloroglucinol-HCl staining of stem sections through the mid-region of wild-type (K) and *broomhead* (L) plants. Phloroglucinol-HCl stains lignified tissue red/pink. Ectopic lignification in the cambial region of *broomhead* plants is indicated by arrows. pi, pith; co, cortex; fc, fascicular cambium; if, interfascicular fibers; pc, phloem cap cells; ps, phloem sieve cells; ifp, interfascicular fiber precursors. Bars = 100  $\mu\text{m}$  in A, B, D to G, K, and L, 50  $\mu\text{m}$  in H to J, and 10  $\mu\text{m}$  in C.

the endogenous *eRF1-1* transcript, it seems likely that the *broomhead* phenotype is a result of cosuppression. In addition to the molecular evidence presented so far, the phenotypic characteristics of the *broomhead* phenotype, such as the variability within and between lines, the low percentage of progeny showing a phenotype, and the differences in the onset of silencing, are also consistent with other reports of transgenic plants showing cosuppression effects (Elkind et al., 1990; Napoli et al., 1990; de Carvalho et al., 1992; Hart et al., 1992; Zhang et al., 1992; Kunz et al., 1996; Palauqui et al., 1996).

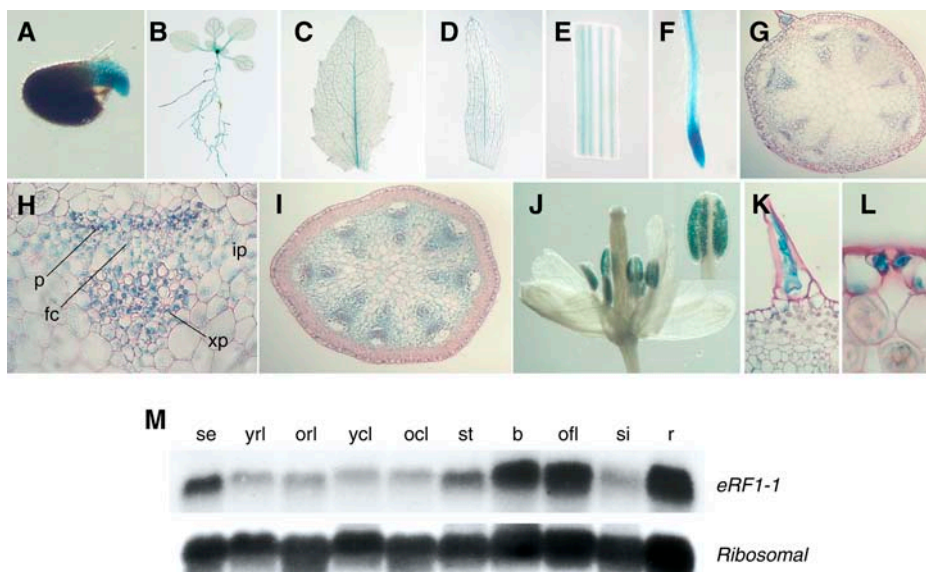
#### Down-Regulation of *eRF1-1* Affects Cell Elongation and Radial Cell Division

By down-regulating an Arabidopsis eukaryotic release factor, we have produced an unexpected yet interesting phenotype that is predominantly defective in inflorescence stem cell elongation. The majority of *broomhead* transgenics resemble wild-type plants in the vegetative stage of development, but during repro-

ductive growth, the inflorescence stems cease to elongate properly, producing a cluster of mostly malformed siliques. Histological analysis on the *broomhead* stem revealed that the clustering of siliques is a result of a reduction in cell elongation. In addition, ectopic lignification of the phloem cap cells, some phloem sieve cells, and regions of the disorganized fascicular cambium was observed as well as enhanced lignification of the interfascicular fibers. This is in contrast to what occurs in wild-type plants, where lignin deposition is limited to xylem and fully elongated interfascicular parenchyma cells (Dharmawardhana et al., 1992; Altamura et al., 2001).

Several Arabidopsis mutants that are defective in cell expansion and display ectopic lignification have been reported (Caño-Delgado et al., 2000; Zhong et al., 2000). The majority contains disruptions in genes involved in cell wall synthesis, such as cellulose synthase (*CESA1* and *CESA3*), endo-1,4- $\beta$ -glucanase, and a chitinase-like gene (Arioli et al., 1998; Nicol et al., 1998; Zhong et al., 2000; Ellis et al., 2002; Caño-Delgado et al., 2003), although disruption of the C-subunit of a putative vacuolar ATPase (Schumacher et al., 1999)





**Figure 8.** *eRF1-1* expression pattern as revealed by staining of *eRF1-1* promoter-*GUS* transgenic lines and northern analysis. *eRF1-1* expression was detected in germinating seeds (A), the vascular tissues of young seedlings (B), rosette leaf veins (C), cauline leaf veins (D), the inflorescence stem (E), roots (F), pollen grains (J), trichomes (K), and guard cells (L). The shoot apical meristem (B) and the root meristem (F) also show GUS activity. G, Transverse section through the mid-region of an inflorescence stem revealing GUS expression is mainly restricted to the vascular bundles and the interfascicular region. H, Enlargement of a vascular bundle from G, displaying GUS expression in phloem (p), fascicular cambium (fc), xylem parenchyma (xp), and interfascicular parenchyma (ip) cells. I, Transverse section through the elongating region of an inflorescence stem showing a more uniform pattern of expression across all cell types. M, Northern blot of total RNA isolated from different tissues (se, seedlings; yrl, young rosette leaves; orl, old rosette leaves; ycl, young cauline leaves; ocl, old cauline leaves; st, stem; b, buds; ofl, open flowers; si, siliques; r, roots) of wild-type plants. The membrane was hybridized with a radiolabeled *eRF1-1* probe and then subsequently stripped and rehybridized with a ribosomal probe to show equal sample loading.

and overexpression of *AtMYB61*, encoding for a transcription factor (Newman et al., 2004), also produced similar phenotypes. As a number of mutants (*eli1-1*, *rsw1-1*, and *det3*) exhibited a reduction in cellulose levels as well as increased levels of defense-related genes, such as *VEGETATIVE STORAGE PROTEIN 1* and *PLANT DEFENSIN 1.2*, it was suggested that changes in cell wall integrity can stimulate lignification and defense responses through jasmonate and ethylene-mediated pathways (Ellis et al., 2002; Caño-Delgado et al., 2003). Despite not having a described role in cell wall synthesis, *eRF1-1* may indirectly affect this process when down-regulated. However, it seems unusual that only specific cell types are ectopically lignified in *broomhead* plants, whereas the majority of ectopic lignification mutants, e.g. *elp1*, *eli1*, and *det3*, exhibit a more random pattern of lignification in the pith and surrounding areas. A possible explanation for this may be that certain cells in *broomhead* plants are more severely affected by a decrease in *eRF1-1* levels than other cell types and, therefore, only stimulate a defense response, resulting in lignin deposition in these cells. The fact that the pith cells are not lignified, yet are inhibited in elongation, may be a consequence of lignification of the surrounding tissues.

In addition to the cell elongation defect, cell division is also affected in *broomhead* plants. In some fascicular cambial regions, cell division is more prolific, although

in the majority of vascular bundles, the cambium exhibits disorganized cell growth. This disorganization of the cambium is also associated with a corresponding enlargement of the nuclei, suggesting that the cell cycle has been disrupted such that DNA replication has become uncoupled from cell division. However, it is also possible that the nuclei appear enlarged because they are decondensed and actively transcribing. The fact that some fascicular cambial regions appear to be more active, while others exhibit disorganized cell growth and contain enlarged nuclei, even within the same stem section, may be directly related to the level of *eRF1-1* down-regulation.

#### Endogenous Expression of *eRF1-1*

The *eRF1-1* promoter-*GUS* experiments revealed that the expression pattern of *eRF1-1* is primarily observed in the vascular tissues and the actively growing and elongating zones of *Arabidopsis* plants. The presence of GUS activity in the meristems and elongating zones appears consistent for a protein involved in translation, as it would be expected that protein synthesis is up-regulated to accommodate for new cell growth in these tissues. However, the observation that GUS activity was mostly restricted to the vascular tissues in the inflorescence stems and leaves was unexpected, although this may be due to a lack of



sensitivity in the GUS staining method. Previously it has been shown that a number of promoter-GUS transgenics exhibit preferential staining in the vascular tissues (Gallagher, 1992). This has been attributed to the presence of smaller, more cytoplasmically dense cells, such as the cambial cells and companion cells in the phloem, which would concentrate the GUS stain, thus leading to the appearance of a stronger expression pattern. However, it is interesting that the areas in the stem that are most affected in *broomhead* plants, i.e. the vascular bundles and interfascicular region, are also the areas where high levels of *eRF1-1* are observed, suggesting that the vascular staining observed in our GUS studies does reflect the true expression pattern of *eRF1-1*. Furthermore, it would make sense that a reduction in *eRF1-1* levels would have the most dramatic effect on cells where it is normally expressed, therefore leading to the morphological aberrations described earlier in the vascular tissues and interfascicular region. The finding that the cells in the apical meristem stained positive for GUS activity but did not appear to be affected in *broomhead* plants may reflect the fact that the apical meristem often escapes the effects of posttranscriptional gene silencing (Voinnet et al., 1998; Foster et al., 2002).

#### The *Broomhead* Phenotype: Disruption of Release Factor Activity?

It has been previously established that a reduction in eRF1 protein levels decreases the efficiency of translation termination and therefore causes an increase in the translational readthrough of proteins (Stansfield et al., 1996). In yeast cells, complete deprivation of eRF1 results in a loss of cell viability (Inge-Vechtsov et al., 2003). Based on these data and the fact that all three Arabidopsis eRF1s have been shown to possess release factor activity (Chapman and Brown, 2004), it seems reasonable to suggest that the *broomhead* phenotype is due to a decrease in the efficiency of translation termination. However, it is interesting to note that eRF1 has also been suggested to perform additional functions, at least in yeast, to that of translation termination. For instance, analysis of multiprotein complexes has revealed that eRF1 is not only associated with other translational components but is also found in complexes containing nontranslational proteins (Gavin et al., 2002; Ho et al., 2002). In addition, eRF1 has also been shown to directly interact with other proteins, some of which have functions outside of translation and could implicate eRF1 in additional processes. These include protein phosphatase 2A, Upf1, Itt1, Mtt1, and a myosin light chain protein (Andjelković et al., 1996; Czapinski et al., 1998, 2000; Uraikov et al., 2001; Valouev et al., 2004).

Further support for an alternative function comes from the various yeast eRF1 mutants that are recessive and have allele-specific pleiotropic effects such as temperature sensitivity (Inge-Vechtsov and Andrianova, 1975; Ter-Avanesyan et al., 1982a), osmotic sensitivity

(Ter-Avanesyan et al., 1982a), and either partial or complete respiration deficiency (Ter-Avanesyan et al., 1982b). A role for eRF1 in yeast cell cycle regulation and cytoskeleton organization has also been suggested by several authors (Tikhomirova and Inge-Vechtsov, 1996; Borkhsenius and Inge-Vechtsov, 1997; Borkhsenius et al., 2000; Stevenson et al., 2001), and inactivation of eRF1 has been associated with myelodysplastic syndrome and acute myeloid leukaemia in humans (Guenet et al., 2000; Dubourg et al., 2002). However, none of these studies were able to conclude whether the phenotypes were due to increased translational readthrough or disruption of eRF1 in an unrelated process.

So far, the most compelling evidence for an additional role for eRF1 has come from a study by Valouev et al. (2002), which demonstrated that the phenotype of an eRF1 yeast mutant was independent of its function in translation. In this study, it was shown that repression of eRF1 resulted in an increase in the level of non-sense codon readthrough and the accumulation of unbudded cells with 2C and higher DNA content, indicating an uncoupling of DNA replication from budding. However, repression of eRF3 (also necessary for the termination reaction) caused a different effect on cell morphology at similar levels of non-sense codon readthrough. This implies that the cellular phenotype of the yeast eRF1 mutant was not due to impairment of the termination process and therefore suggests a role for eRF1 in cytoskeleton organization and cell cycle regulation. The similarity between this study and our observations, which showed enlarged nuclei in the disorganized fascicular cambial cells, further adds to the possibility that eRF1 may function in other processes.

In conclusion, we have established that cosuppression of *eRF1-1* in Arabidopsis has a dramatic effect on plant morphology, ultimately causing a reduction in internode elongation. The *broomhead* phenotype is the first report of any functional characterization of a release factor in vivo in Arabidopsis. However, the specific effects down-regulating eRF1 has on certain cell types, e.g. the cambial cells and the ectopically lignified cells, allow for speculation as to whether Arabidopsis eRF1-1 also functions in additional processes to that of translation termination. Examining the subcellular localization of eRF1-1, measuring the ploidy levels in *broomhead* plants, and screening for interacting proteins in Arabidopsis will provide further insight into the functions of eRF1-1 and may establish whether or not translation termination is its only role.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

For all phenotypic analyses, Arabidopsis (*Arabidopsis thaliana*) seeds were sown on soil and stratified for 2 to 5 d at 4°C. Trays of seed were then transferred to a temperature-controlled growth room and grown at 21°C under 50  $\mu\text{E}/\text{m}^2/\text{s}$  of long-day light (16/8 light/dark).

## Plasmid Constructs

The *eRF1-1* overexpression construct was produced by inserting the full-length *eRF1-1* cDNA (GenBank accession no. H37361) into the *EcoRI* and *BamHI* sites of the binary vector pSOV<sub>2</sub> (Mylne and Botella, 1998). The *eRF1-1:GUS* fusion was constructed by amplifying the coding sequence of *eRF1-1* using the primers 5'-CGTCTAGAATGGGAGACAAAAACGATGAC-3' and 5'-CGGGATCCCCTCCGAATCATCATCAAGAGC-3' and cloning the fragment in frame with the *GUS* coding region into the *XbaI* and *BamHI* sites of the binary vector pAOV-*intronGUS*. In the resulting construct, *eRF1-1* is fused to the N-terminal region of *GUS*. pAOV-*intronGUS* is derived from the vector pAOV (Mylne and Botella, 1998) and has the *GUS* gene containing an intron as described by Ohta et al. (1990) cloned into the *BamHI* and *SacI* sites of the vector.

*eRF1-1*, *eRF1-2*, and *eRF1-3* promoter-*GUS* fusions were generated by cloning approximately 2 kb of sequence upstream of each of the translational start sites into the promoterless vector pAOV-*intronGUS*Δ35S. This vector is based on pAOV-*intronGUS* but lacks the CaMV 35S promoter. The primers used to clone all three promoter regions are as follows: *eRF1-1*, 5'-CGCTCGAGGGCTGATATGCGACAACCATAAAG-3' and 5'-CGAAGC-TTCTTAACAATAGAACAAAAAC-3'; *eRF1-2*, 5'-CGAAGCTTCTTAGTT-TATTGACCAGCCAGTTG-3' and 5'-CGAAGCTTTTTAGCTTCTCTCGAG-GTCC-3'; *eRF1-3*, 5'-CGCTCGAGCTGGCTACCGAAGTCCGCGTAAG-3' and 5'-CGATCGATGTTTTCTCCTTCAAGC-3'.

Wild-type *Arabidopsis* plants (ecotype Columbia) were transformed using the floral dip method (Clough and Bent, 1998) and selection of T<sub>1</sub> seeds was achieved by spraying seedlings with 0.4% BASTA (Hoechst).

## RNA Analysis

Tissue samples were collected and immediately frozen in liquid nitrogen. RNA was extracted as described by Etheridge et al. (1999). Northern analysis was performed with 10 μg (per lane) of total RNA using standard procedures (Sambrook et al., 1989). After the initial hybridization, all blots were stripped and rehybridized with a wheat ribosomal probe (Gerlach and Bedbrook, 1979) to test for equal loading. Hybridization was performed in Church buffer (Church and Gilbert, 1984) at 65°C overnight, and the blots were washed at high stringency in 2× SSC, 0.1% SDS for 15 min at room temperature, then in 2× SSC, 0.1% SDS for 15 min at 55°C, and finally in 0.2× SSC, 0.1% SDS for 15 min at 55°C.

## Southern Analysis

Genomic DNA was isolated from 2-week-old *broomhead* seedlings as previously described (Etheridge et al., 1999). Three micrograms of DNA was digested with *EcoRV*, *SalI*, or *HindIII*, and samples were electrophoresed on a 0.8% agarose gel, then transferred to a Hybond-N<sup>+</sup> nylon membrane (Amersham Biosciences) by capillary transfer (Sambrook et al., 1989). The blot was then hybridized with an *eRF1-1* radiolabeled DNA probe and washed at high stringency as described for the northern analysis.

## Quantitative Real-Time PCR

Three biological replicates were performed on three independent *broomhead* lines. Total RNA was extracted from bud tissue (Promega SV Total RNA Extraction kit), and 1 μg was used to synthesize cDNA using reverse transcriptase and random hexamers in a reaction volume of 20 μL. Prior to quantitative PCR, cDNA samples were diluted 5-fold in water and then amplified using the ABI PRISM 7700 sequence detector and SYBR Green Master mix (Applied Biosystems). Gene-specific primers were designed to regions of low homology between the gene family members using Primer Express 1.5 software (Applied Biosystems). Primers used and locus identifiers are as follows: *eRF1-1* forward, 5'-ACTGCCTTTGATTCCGAGGA-3'; *eRF1-1* reverse, 5'-GCCATGGTGAGGATTTGATTG-3'; *eRF1-2* forward, 5'-TTGAT-TTCTCCTTCTCCATCTTCG-3'; *eRF1-2* reverse, 5'-CAGATCTCGATGTTCCG-TATCCG-3'; *eRF1-3* forward, 5'-TTAAGAACAACACGACCGGAGA-3'; *eRF1-3* reverse, 5'-GCATCATGGAATGCTTTGGT-3'. Locus IDs are as follows: *eRF1-1* (At5g47880), *eRF1-2* (At1g12920), *eRF1-3* (At3g26618). Expression detected from three *β*-actin genes of *Arabidopsis*, *β*-actin-2 (At3g18780), *β*-actin-7 (At5g09810), and *β*-actin-8 (At1g49240) with universal actin forward primer 5'-AGTGGTCTGACACCGGIATTGT-3' and specific reverse primers 5'-GATGGCATGGAGGAAGAGAGAAAC-3', 5'-GAGGAAGAGCAT-

TCCCCTCGTA-3', and 5'-GAGGATAGCATGTGGAAGTACTGAGAA-3', respectively, were used as combined internal standards to normalize small differences in template amounts.

## Histological Analysis

Stem sections from areas of reduced internode elongation in *broomhead* plants and the corresponding regions in wild-type plants were selected and fixed in ethanol:acetic acid (3:1) overnight. Samples were then dehydrated through a tertiary-butyl alcohol series, and flakes of Paraplast plus (Sigma-Aldrich) were added to the final step. Once the flakes dissolved at room temperature, liquefied Paraplast plus was added, and samples were transferred to an oven at 58°C. The medium was replaced after approximately 8 h, and samples were put back in the oven overnight. Stem samples were positioned in Paraplast plus, and microtome sections of 10 μm were cut, mounted on slides, and stained with safranin-O and fast green. Stained sections were photographed under bright-field illumination. To specifically detect the presence of lignin, deparaffinized and rehydrated tissue sections were stained for 5 min with phloroglucinol (2.5% [w/v] phloroglucinol in 6 N HCl) and rinsed in water. The slides were then mounted in 50% glycerol, 6 N HCl and visualized under a light microscope.

## Detection of GUS Activity

Whole plant specimens and small tissue sections were incubated at room temperature in chloroform for 15 min, then rinsed approximately two to three times in rinse solution (staining solution lacking 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). GUS stain (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 4 mM potassium ferro-/ferricyanide, 0.1% Silwet L-77, and 100 mM sodium phosphate buffer, pH 7.0) was added and allowed to vacuum infiltrate the tissue for 15 min, twice. Samples were then incubated at 37°C overnight and destained in 70% ethanol the following day. Stem tissue for sectioning was transferred to ethanol:acetic acid (3:1) after staining in the GUS solution and then prepared in the same way as samples described for histological analysis. Microtome sections of 15 μm were mounted on slides and stained with safranin-O.

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