The REIL1 and REIL2 Proteins of Arabidopsis thaliana Are Required for Leaf Growth in the Cold1[W][OPEN]

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The evolutionarily conserved proteins REI1-LIKE (REIL1) and REIL2 have four conserved zinc finger domains and are Arabidopsis thaliana homologs of the cytosolic 60S ribosomal maturation factor Rei1p (for Required for isotropic bud growth1 protein) from yeast (Saccharomyces cerevisiae) and its paralog Reh1p (for REI1 homologue1 protein). The yeast and A. thaliana paralogs result from independent gene duplications. The A. thaliana REIL paralogs are required specifically in the cold (10°C) but not for growth at optimal temperature (20°C). A reil1-1 reil2-1 double mutant is arrested at 10°C prior to the emergence of the first rosette leaf. Two allelic reil2 mutants, reil2-1 and reil2-2, form small spoon-shaped leaves at 10°C. This phenomenon reverts after emergence of the inflorescence in the cold or upon shift to 20°C. Except for a slightly delayed germination, a reil1-1 mutant shows no further growth phenotype under the currently investigated conditions. A comparative analysis demonstrates conserved coexpression of orthologous genes from yeast and A. thaliana that are coregulated with yeast rei1 or with A. thaliana REIL2, respectively. The conserved correlations point to a role of A. thaliana REIL proteins in the maturation of the eukaryotic ribosomal 60S subunit. We support this conclusion by heterologous complementation of the cold-induced growth defect of the yeast rei1 deletion.

Genes coding for zinc finger proteins are abundant features of plant, fungal, and animal genomes (Riechmann et al., 2000). Approximately 0.8% of all proteins from the budding yeast (Saccharomyces cerevisiae) have zinc finger domains (Böhm et al., 1997). Plant genomes harbor similar proportions of zinc finger proteins. The eudicot model plant Arabidopsis thaliana, for example, has 176 zinc finger proteins (Englbrecht et al., 2004; Ciftci-Yilmaz and Mittler, 2008). A family of 189 zinc finger proteins is encoded by the genome of the monocot model Oryza sativa (Agarwal et al., 2007). Most of the members of these large protein families are plant-specific transcriptional regulators. Plant zinc finger proteins contain one or more typical C2H2-type zinc finger domains but may also have variations of this motif (Englbrecht et al., 2004; Agarwal et al., 2007; Ciftci-Yilmaz and Mittler, 2008). Only 32 proteins of A. thaliana contain four zinc finger domains. The four zinc finger proteins of this study, i.e. the REI1-LIKE protein REIL1 encoded by At4g31420 and the REIL2 paralog encoded by At2g24500, represent a small gene family and belong to the few (18.8%) evolutionarily conserved zinc finger proteins (Englbrecht et al., 2004; Ciftci-Yilmaz and Mittler, 2008).

In agreement with the predominant function of zinc finger domains, both A. thaliana REIL genes are currently annotated as putative sequence-specific DNA-binding transcription factors (http://www.arabidopsis.org). Even though most plant zinc finger proteins are transcription factors, the ligand range of the zinc finger motif is known to have diversified during evolution. Zinc fingers that specifically interact with proteins (Gamsjaeger et al., 2007) or with RNA, e.g. ribosomal RNA, mRNA, and poly(A)-RNA, have been described (Hall, 2005; Kelly et al., 2007).

Contraindicative to the current provisional functional annotation of A. thaliana REIL proteins, the zinc finger motifs of these proteins are reported to show similarity to U1-zinc fingers (Englbrecht et al., 2004). These motifs form characteristic domains of the U1 small nuclear ribonucleoproteins, which bind to the 5' splice site of pre-mRNA in the course of spliceosome assembly. In view of this conflicting evidence, we considered the REIL proteins of A. thaliana at the beginning of our study to represent true orphans without functional assignment.

Attempts to deduce the function of plant REIL genes by previously characterized orthologs of other taxa yield conflicting results. Several clusters of orthologs (COGs) are reported that contain the A. thaliana REIL proteins (Tatusov et al., 2003; O’Brien et al., 2005; Kuzniar et al., 2009; Östlund et al., 2010). The members of these COGs are exclusively of eukaryotic origin and comprise proteins of plants, fungi, nematodes, insects, and mammals. With only few exceptions, REIL orthologs are not functionally characterized. One of these exceptions is a single-copy mammalian protein. This protein is named Zinc Finger-Like Protein9 (ZRP9; Seong et al., 2002, 2003) and is also known as zinc finger protein 622 (ZNF622). The second exception is a set of two functionally characterized paralogs from yeast, namely Rei1p...
According to a mitotic phenotype of the to belong to the mitotic signaling network of yeast. Describes Rei1p as a cytoplasmic protein that appears phosphorylated by ASK1 at the positions S314 and T318 (Seong et al., 2011). The apoptosis signal-regulating kinase1 (ASK1) and is in phosphosite.org). ZRP9 is also a positive regulator of apoptosis of the human ZRP9 protein sequence (http://www.phosphosite.org). ZRP9 is also a positive regulator of apoptosis signal-regulating kinase1 (ASK1) and is in turn phosphorylated by ASK1 at the positions S314 and T318 (Seong et al., 2011).

The initial study on yeast Reilp and its paralog Rehlp also points toward an involvement in cell proliferation processes (Iwase and Toh-e, 2004). This study describes Rei1p as a cytoplasmic protein that appears to belong to the mitotic signaling network of yeast. According to a mitotic phenotype of the ∆rei1 deletion mutant, Rei1p is named for being required for isotropic bud growth (Iwase and Toh-e, 2004). This line of investigation was, however, not further pursued when two laboratories demonstrated that Rei1p takes part in the maturation process of the 60S preribosomal subunit. In more detail, Rei1p interacts with the cytosolic shuttling factors Associated with ribosomal export complex1 (Arx1p), ARX1 little brother1 (Alb1p), Ribosomal-like protein24 (Rlp24p), ribosomal protein of the large subunit24A (Rpl24Ap), Rpl24Bp, Type III j-protein1 (Jj1p), and translation initiation factor6 (Tif6p) (Hung and Johnson, 2006; Lebreton et al., 2006; Meyer et al., 2007). Reilp is thought to be recruited to the cytosolic pre-60S subunit by Rlp24p. Rlp24p needs to be removed before Reilp can load onto the cytosolic 60S preribosome. Rlp24Ap or the almost-identical Rlp24Bp are thought to replace Rlp24p in the cytosol. Jj1p is an Hsp40 heat shock chaperone (Dempoinet et al., 2007) and cooperates with Rei1p to remove Alb1p from the pre-60S subunit (Meyer et al., 2010). This step initiates Arx1p and Alb1p recycling to the nucleus. Arx1p release, in turn, is thought to be prerequisite of the final releases of Tif6p (Basu et al., 2001) and Nonsense-mediated mRNA decay3 protein (Nmd3p) from the pre-60S subunit (Lo et al., 2010). These processes form the final control point that renders the cytosolic pre-60S ribosomal subunits of yeast mature and translationally active. While Reilp is central to the mechanism of Arx1p-dependent 60S subunit maturation, the paralog Rehlp appears to stabilize the 60S subunits in the absence of Reilp and may have a partially redundant function. Rehlp possibly takes part in a basic 60S subunit maturation process, which is thought to be independent of Arx1 recycling (Parnell and Bass, 2009). These processes were discovered in yeast and can currently be considered to represent the mechanistic model of the final steps of eukaryotic 60S ribosomal maturation (Lo et al., 2010; Panse and Johnson, 2010).

Growth phenotypes of the yeast ∆rei1, ∆reh1, and double deletion mutants link Reilp function to abiotic stress response mechanisms of this organism. Reilp is required to maintain growth when yeast is shifted to even moderately suboptimal temperature conditions. Growth of the ∆rei1 mutant is reduced 2- to 3-fold at 23°C to 25°C but normal at optimal 30°C conditions (Iwase and Toh-e, 2004; Lebreton et al., 2006). By contrast, Rehlp is not required for growth at low temperature. However, the double deletion mutant is growth arrested already at temperatures below or equal to 30°C and can only be maintained at 37°C (Parnell and Bass, 2009). Gene expression of reil but not of reh1 is under temperature control and exhibits almost thermometer-like, reciprocal responses to high- and low-temperature stresses. In detail, reil mRNA is early up-regulated when cells are shifted from 28°C to 10°C and likewise early down-regulated upon shift from 28°C to 37°C (Strassburg et al., 2010; Walther et al., 2010).

Based on the evidence summarized above, we investigated whether the function of the a. thaliana is conserved. For this purpose, we isolated transfer DNA (T-DNA) mutants of the a. thaliana REIL genes and tested their growth response to suboptimal temperature. In addition, we demonstrate conserved function by comparative coexpression analysis and functional complementation of the ∆rei1 mutant.

RESULTS

The Topology of A. thaliana REIL Proteins

The A. thaliana REIL genes have five exons, which encode a 404-amino acid REIL1 protein, gene model At4g31420.1, and a 395-amino acid REIL2 protein, gene model At2g24500.1 (Fig. 1A). REIL1 has a putative splice site variant, At4g31420.2, which adds an additional Ser within ZF4, sites S276; Supplemental Document S1). An ortholog translated from the genomic sequence of Thellungiella halophila and Arabidopsis lyrata ssp. lyrata, and Thellungiella halophila (Supplemental Figs. S1 and S2; Supplemental Document S1). An ortholog translated from the genomic sequence of Brassica rapa ssp. pekinensis aligns to REIL1. A partial sequence of the same subspecies that aligns to REIL2 indicates the presence of a second paralog in Brassica rapa ssp. pekinensis (Supplemental Fig. S2). The four zinc finger domains of REIL proteins are positioned in two pairs, ZF1/ZF2 and ZF3/ZF4, at the N terminus and in the center of the sequence. Two additional highly conserved domains, here provisionally termed CD1 and CD2, are located close to and at the C terminus (Fig. 1A). The phosphorylation sites of the human ortholog ZRP9 (Seong et al., 2011) are located within a highly conserved region adjacent to ZF3, sites S276 and Y284 of ZRP9, and within ZF4, sites S314 and T318 of ZRP9. Only one of the phosphorylation sites, Y284, is conserved in the REIL proteins of the Brassicaceae. The eukaryotic orthologs
Figure 1. Topology and phylogeny of the REIL proteins from *A. thaliana*. A, Topology of *A. thaliana* REIL genes and proteins. The *REIL1* and *REIL2* genes have five conserved exons. The respective REIL proteins, namely the 404-amino acid REIL1 and the 395-amino acid REIL2 protein, contain four zinc finger domains and two additional conserved domains, CD1 and CD2. The mutants, *reil1-1* (SALK_090486), *reil2-1* (GK_166C10), and *reil2-2* (SALK_040068), carry T-DNA insertions in exon 2 (compare with arrow heads). B, Phylogenetic analysis of the plant REIL proteins. The plant REIL proteins were subject to gene duplications, which occurred independently in several plant phyla. The REIL1 and the REIL2 paralogs of *A. thaliana* originated from gene duplication during the speciation of the Brassicales.
of the *A. thaliana* REIL proteins are mostly single copy (Supplemental Fig. S3; Supplemental Table S1). Gene duplications occurred independently during evolution of the Saccharomycetales and of the Embryophyta. None of the species with full genome information has more than two paralogs.

The origin of the yeast reil1 and reil1 paralogs appears to be linked to a gene duplication following the phylogenetic ancient speciation of the Hemiascomycete *Yarrowia lipolytica* (Dujon et al., 2004; Dujon, 2006). *Y. lipolytica* contains only one reil1-like copy, whereas modern yeast species have two (Supplemental Fig. S3). Species of the plant phylum, except for the monocots, tend to have independent gene duplications of the reil1-like genes (Fig. 1B). In addition to the gene duplication, which gave rise to the REIL1 and REIL2 paralogs of the *A. thaliana* like genes (Fig. 1B). In addition to the gene duplication, which gave rise to the REIL1 and REIL2 paralogs of the Brassicaceae, gene duplications are present within the *Physcomitrella patens* and *Selaginella moellendorffii* genomes, as well as in *Populus trichocarpa* and *Malus domestica*.

### The *A. thaliana* reil Mutants

To match the yeast gene deletion mutants, we isolated a *reil1-1* mutant and two allelic *reil2* mutants of *A. thaliana*, namely *reil2-1* and *reil2-2*, from public T-DNA insertion mutant collections. The selected T-DNA insertions of *reil1-1*, *reil2-1*, and *reil2-2* were in exon 2 at base pairs 475, 733, and 731, respectively. All tests of the mutants for the presence of full-length mRNA, both before and after flowering and at 10°C as well as at 20°C (Supplemental Fig. S4), were negative. The homozygous single gene mutants and a homozygous *reil1-1 reil2-1* double mutant were fully viable under optimal growth conditions in a 16-h/8-h day/night cycle at 20°C/18°C. The mutant plants propagated under these conditions without obvious defect compared to the ecotype Columbia-0 (Col-0) wild type. To avoid differential priming effects on seed batches, all seed material of this study was from plants that completed a full life cycle under optimum growth conditions. The germination rates of these mutant seed batches were not significantly changed compared with a 90.1% ± 2.8% germination rate of the wild type (Supplemental Table S2).

### Characterization of the Mutant Phenotypes: the reil1-1 reil2-1 Double Mutant Has Pointed-Leaves Morphology

Germination of the *reil* mutant lines was assayed in vitro under an optimum 20°C temperature regime and staged according to a system for the phenotypic analysis of *A. thaliana* development (Boyce et al., 2001). The emergence of the first pair of rosette leaves, stage 1.02 (Fig. 2), was delayed in the *reil1-1 reil2-1* double mutant but not the emergence of the radicle at stage 0.5 or the emergence of the cotyledons at stage 0.7 (data not shown). The *reil1* and *reil2* single gene mutants did not differ from the wild type at 20°C (Fig. 2). In contrast to the wild type and the single gene mutants, the leaf morphology of early leaves generated by the *reil1-1 reil2-1* double mutant was aberrant (Fig. 3, A and B). These developing leaves were spear shaped, with an acute tip and two basal serrations instead of the typical rounded leaves of the Col-0 wild type. The aberrant leaf morphology was similar to the so-called pointed-leaves mutations (Van Lijsheettens et al., 1994; Berná et al., 1999; Horiguchi et al., 2011). The pointed-leaves phenotype of the *reil1-1 reil2-1* double mutant gradually disappeared at later growth stages and was lost after transfer to soil.

#### The reil1-1 reil2-1 Double Mutant Is Growth Arrested in the Cold

Lowering temperatures delayed the germination process of the *reil1-1 reil2-1* double mutant further and enhanced the pointed-leaves morphology. A complete growth arrest was reached at 10°C (Fig. 2). At 10°C, the seedlings of the *reil1-1 reil2-1* double mutant generated roots and cotyledons with a delay of approximately 3 to 4 d compared with the wild type and did not develop beyond stage 1.0 (Boyce et al., 2001). Upon prolonged exposure to cold, the double mutant bleached and accumulated a violet tinge (Fig. 3B). When transferred to soil, the seedlings stayed arrested, did not generate rosette leaves, and ultimately died in the cold (Fig. 3C). The *reil1-1* mutant produced the first rosette leaf with only a slight delay and was otherwise inconspicuous during the germination assays and the subsequent morphological assessments. By contrast, the germination of both *reil2* mutants was delayed at 10°C (Fig. 2). Germination and cultivation at 4°C was attempted, as this temperature is the typical choice of cold stress cues applied to *A. thaliana*. Our experiments failed to produce precise results at 4°C due to slow and variable germination of the *reil* mutant seedlings. For this reason, we choose 10°C as the standard cold stress condition of this study.

#### Leaves of the *reil2* Mutants Have an Aberrant Shape That Reverts after Continued Cultivation in the Cold

In contrast to the double mutant, the *reil2* mutants stayed viable at 10°C. The leaf morphology of both the *reil2-1* and the *reil2-2* mutants was irregular during cold germination. The leaves had a corrugated adaxial and a smooth abaxial surface and became spoon shaped upon transfer to soil and continued cultivation at 10°C (Fig. 3). However, the aberrant shape of *reil2-1* and *reil2-2* leaves reverted after continued cultivation at 10°C (Fig. 4, A and B). Morphometric analysis of the *reil2* mutants showed a significant growth deficit in the cold compared with the wild type and the inconspicuous *reil1-1* mutant (Fig. 5). The growth deficit was apparent in all monitored aspects, namely leaf number, planar rosette area, and surface coverage, i.e. the percentage of the planar leaf area within the convex hull of a rosette.
The growth deficit was maintained throughout development in the cold (Fig. 5, left). Between week 5 and 6 on soil in the cold, the leaf shape of the rei2 mutants reverted to a shape that was similar to the wild type. Reversion included longitudinal leaf growth and coincided with the emergence of the first floral buds (Figs. 4B and 5). The transition between the two leaf morphologies was best represented by the surface coverage of the rosette (Fig. 5). The wild type and rei1-1 mutant had almost constant surface coverage throughout development in the cold. By contrast, the rei2 mutants started with lower surface coverage, reached a maximum higher than the wild type at 6 weeks, and then reverted to lower surface coverage. The rei2-1 mutant had a slight but significantly stronger growth deficit compared with the rei2-2 mutant (Fig. 5).

The Aberrant Shape of rei2 Leaves Is Lost after Shift to Optimal Temperature

The aberrant shape of rei2-1 and rei2-2 leaves reverted also after shift to optimum cultivation conditions at 20°C (Fig. 4C). However, the growth deficit compared with the wild type was maintained. The rei2-1 mutant was again more affected than the rei2-2 mutant (Fig. 5, right). The trends of changes of the rei2 surface coverage were essentially the same as in prolonged cold, but more rapid. Less leaves with longer petioles and lower surface coverage
were generated after the shift to 20°C (Figs. 4C and 5). The main difference between the two modes of reversal was the elongation of the leaf lamina in prolonged cold, in contrast to a continually stunted and close-to-circular leaf lamina after shift to 20°C.

The Expression of REIL2 and Yeast rei1 Is Controlled by Temperature

Our own and the other studies had shown that the expression of the yeast rei1 and reh1 genes was under differential temperature control (Gasch et al., 2000; Sahara et al., 2002; Strassburg et al., 2010; Walther et al., 2010). The rei1 gene was up-regulated after cold stress and down-regulated after application of heat; the second yeast paralog, reh1, was not temperature responsive (Supplemental Fig. S5). As a consequence, only the rei1 paralog of yeast met the requirement of coexpression analysis. The *A. thaliana* REIL paralogs showed a similar differential response to temperature cues. REIL1 transcript levels were only slightly influenced by temperature (Kaplan et al., 2007; Kilian et al., 2007; Caldana et al., 2011). By contrast, the REIL2 transcript levels were clearly up-regulated in the cold but not significantly changed by heat (Supplemental Fig. S5). REIL2 responded 3 h after the cold cue and was delayed compared with the immediate response of yeast rei1 within the first minutes of cold stress. Again, only one paralog, REIL2, was amenable to coexpression analysis. Notably, the paralogs of both species, which were required for normal growth in the cold, namely REIL2 and rei1, were both under transcriptional control.

Figure 3. The aberrant leaf phenotypes of the reil1-1 reil2-1 double mutant and of the reil2-1 mutant. A, Pointed-leaves morphology of the reil1-1 reil2-1 double mutant compared with Col-0 after 16 d at 20°C. B, Growth arrest of the reil1-1 reil2-1 double mutant at stage 1.0 (Boyés et al., 2001) after 27 d at 10°C compared with Col-0. C, Phenotype of the reil1-1 reil2-1 double mutant germinated at 10°C, transferred to soil, and kept for 10 weeks strictly at 10°C compared with the Col-0 wild type. D, Representative phenotype of the reil2-1 mutant germinated at 10°C, transferred to soil, and kept for 5 to 6 weeks strictly at 10°C compared with the Col-0 wild type.

Figure 4. Phenotype of the reil1-1, reil2-1, and reil2-2 mutants compared with the Col-0 wild type under diverse temperature regimes. A, Phenotype after germination at 10°C, transfer to soil at stage 1.02 to 1.03, and continued growth at 10°C for a period of 4 weeks. B, Phenotype after germination at 10°C, transfer to soil at stage 1.02 to 1.03, and continued growth at 10°C for a period of 8 weeks. C, Phenotype after germination at 10°C, transfer to soil at stage 1.02 to 1.03, continued growth at 10°C for a period of 2 weeks, and subsequent shift to 2 weeks at 20°C. Note reversal of the reil2 leaf phenotype and emergence of inflorescences from all mutant and wild-type rosettes. C, Phenotype after germination at 10°C, transfer to soil at stage 1.02 to 1.03, continued growth at 10°C for a period of 2 weeks, and subsequent shift to 2 weeks at 20°C. Note reversal of the reil2 leaf phenotype after shift to optimum temperature. All photographs have equal scale. Vertically aligned photographs were taken with identical illumination.
None of the above conditions caused a growth arrest of the double mutant or a growth defect of either the reil1 or the reil2 single mutants. To test for more subtle changes of growth rate, we choose an in vitro vertical plate assay and scored the length of the primary root of young A. thaliana plantlets. Under control conditions, the single gene mutants and the Col-0 wild type had identical root growth rates, approximately 6.6 mm day\(^{-1}\). Only the root growth of the reil1-1 reil2-1 double mutant was reduced to 4.7 mm day\(^{-1}\). In each case, root growth was linear between 7 and 14 d after germination (Supplemental Fig. S6). We defined the sensitivity to a stress factor as the ratio of the growth rate of each genotype under stress conditions divided by the growth rate of the same genotype under control conditions (Supplemental Fig. S7). The reil mutants and Col-0 were mostly equally sensitive to the tested stress factors. Only minor changes were observed compared with the complete growth arrest of the reil1-1 reil2-1 double mutant at 10°C. Specifically, the reil2 mutants were slightly more sensitive to high light and to oxidative stress induced by paraquat (Supplemental Fig. S7). These results supported the specificity of the growth defect of the reil mutants under cold stress.

**A. thaliana REIL2 and Yeast reil Have Similar Coexpression Patterns**

To test for a conserved function of A. thaliana REIL genes, we compared the coexpression patterns between the temperature-controlled yeast reil1 and A. thaliana REIL2 isoforms. The preceding specificity test of the mutant phenotype allowed us to focus our coexpression analysis on temperature stress experiments only, rather than to perform an unspecific coexpression analysis based on all available diverse stress response data. To take into consideration that yeast reil1 is controlled by cold and heat stress while A. thaliana REIL2 responds only to cold, we performed two parallel analyses. First, we investigated cold-controlled coexpression using only the cold stress data from each of the selected transcript studies. Second, we analyzed the temperature-controlled coexpression modules combining both the cold and the heat stress data from each of the selected transcript studies. Coexpression was analyzed by calculation of rank correlation coefficients. This procedure sorts the numerical expression data of each gene from small to large and assigns ranks, i.e. 1, 2, 3, etc., to each expression value. As a consequence, the rank correlation coefficients are less influenced by outliers, i.e. single very high or very low expression values (Steinhauser et al., 2004). Taken together, we distinguished between four modes of coexpression, namely positive and negative correlation under either cold or temperature control.

Our coexpression analysis demonstrated firstly that REIL2 is positively coregulated with genes that are phylogenetically conserved between A. thaliana and yeast (Supplemental Fig. S8). By contrast, no such enrichment was demonstrated for the negatively coexpressed genes. Secondly, an overrepresentation analysis demonstrated
that REIL2 was positively coexpressed with genes involved in translation initiation and protein synthesis. Genes of the eukaryotic ribosomal subunits were highly significant and robustly enriched in all three analyzed transcriptome studies both under cold and temperature control (Tables I and II). Plant photosystem genes, which are obviously not conserved in yeast, were overrepresented among genes that were negatively coexpressed with REIL2. Furthermore, we found that A. thaliana genes with a positive correlation to REIL2 had yeast orthologs that were also positively correlated to rei1. This coexpression pattern was conserved between species both under cold and temperature control, but more significantly under temperature control (Supplemental Fig. S9). The pairs of A. thaliana / yeast orthologs with conserved coexpression to REIL2 and rei1, respectively, are listed in Supplemental Table S3. This list contains genes of all major steps in ribosomal maturation, including the cytosolic maturation machinery (compare with “Discussion”).

The A. thaliana Orthologs of the Cytosolic Maturation Machinery of the 60S Ribosomal Subunit Show Conserved Coexpression

In yeast, rei1, the homolog of A. thaliana REIL, has a well-characterized function as part of the cytosolic maturation machinery of the 60S ribosomal subunit (Lo et al., 2010). Most of the yeast genes involved in this process had A. thaliana orthologs, which were, previous to our study, not annotated. We show the currently suggested scheme of cytosolic 60S maturation in yeast and the results of our homology searches (Fig. 6).

The yeast genes ATPase family gene2, nucleolar G-protein gene1, rp24, arc1, yeast vaccinia virus VHI homolog, mRNA Turnover4, elongation factor-like1, tif6, large-subunit GTPase1, and the previously mentioned nmd3 were robustly coexpressed with yeast rei1 both under temperature and cold control (Supplemental Table S4). This coexpression pattern was strongly conserved in the A. thaliana nmd3 ortholog At2g03820 (Fig. 6). Moreover, the tif6, Shwachman-Bodian-Diamond syndrome protein ortholog1, and mRNA Turnover4 orthologs of A. thaliana were robustly coexpressed with REIL2 either under cold or under temperature control (Fig. 6; Supplemental Table S4).

The comparison of the complete correlation matrix of the yeast genes involved in cytosolic 60S ribosomal maturation to the complete correlation matrix of their A. thaliana homologs demonstrated further conservation of the coexpression patterns (Fig. 7). Instead of considering only the top-scoring significant correlations, we compared the averages across the respective independent experiments so as to also evaluate conserved absence of coexpression. The temperature- and cold-controlled correlation matrices of yeast were almost identical (Fig. 7; Supplemental Fig. S10). Most of the yeast genes were

| Table I. Significance values of the overrepresentation analysis of functional annotations among genes that are coexpressed with REIL2 under cold control or under temperature control |
|---|---|---|---|---|
| Bin | Data Set | Cold-Controlled Coexpression | Temperature-Controlled Coexpression |
| Bin Name | Caldana et al. (2011) | Kaplan et al. (2007) | Kilian et al. (2007) | Caldana et al. (2011) | Kaplan et al. (2007) | Kilian et al. (2007) |
| Size | P Value<sup>a</sup> | Size | P Value<sup>a</sup> | Size | P Value<sup>a</sup> |
| Negative coexpression | | | | | | |
| 1 | PS | 187 | 4.30E-07 | 3.76E-08 | 1.30E-18 | 1.26E-02 | 1.40E-03 | 2.70E-07 |
| 1.1 | PS.lightreaction | 146 | 3.90E-07 | 4.28E-02 | 5.62E-09 | 2.93E-06 | 8.50E-03 | 7.01E-02 |
| 11 | lipid metabolism | 375 | 4.88E-02 | 7.69E-03 | 1.08E-02 | 1.51E-01 | 2.91E-03 | 1.22E-03 |
| 27.3.5 | RNA.regulation of transcription. ARR | 23 | 8.20E-03 | 2.01E-02 | 4.89E-02 | 6.92E-01 | 4.30E-01 | 1.53E-01 |
| 27.3.6 | RNA.regulation of transcription. bHLH, Basic Helix-Loop-Helix family | 93 | 1.11E-02 | 5.43E-03 | 1.98E-03 | 2.87E-01 | 1.69E-01 | 2.82E-02 |
| Positive coexpression | | | | | | |
| 27.1 | RNA.processing | 254 | 3.26E-04 | 3.86E-10 | 1.13E-06 | 5.30E-01 | 1.36E-02 | 1.03E-13 |
| 27.1.2 | RNA.processing,RNA helicase | 29 | 4.56E-03 | 3.28E-04 | 4.89E-02 | 5.82E-02 | 8.26E-03 | 5.72E-02 |
| 29.2 | protein.synthesis | 515 | 7.73E-13 | <1.0E-20 | <1.0E-20 | 1.0E-06 | <1.0E-20 | <1.0E-20 |
| 29.2.1 | | 371 | 5.94E-05 | <1.0E-20 | <1.0E-20 | 1.68E-07 | 2.37E-08 | <1.0E-20 | <1.0E-20 |
| 29.2.1.2 | | 236 | <1.0E-20 | <1.0E-20 | <1.0E-20 | 1.0E-06 | <1.0E-20 | <1.0E-20 |
| 29.2.1.2.1 | protein.synthesis,ribosomal protein. eukaryotic.40S subunit | 88 | 1.80E-08 | 5.57E-09 | 9.74E-08 | 5.20E-07 | <1.0E-20 | <1.0E-20 |
| 29.2.1.2.2 | protein.synthesis,ribosomal protein. eukaryotic.60S subunit | 148 | 2.06E-12 | <1.0E-20 | <1.0E-20 | <1.0E-20 | <1.0E-20 | <1.0E-20 |
| 29.2.2.50 | protein.synthesis,ribosomal protein.BRIX | 6 | 4.69E-03 | 7.59E-03 | 2.75E-03 | 3.25E-03 | 2.94E-03 | 1.56E-03 |
| 29.2.3 | protein.synthesis.initiation | 84 | 5.59E-08 | 6.44E-11 | 4.15E-11 | 7.73E-07 | 1.82E-07 | 2.94E-10 |

<sup>a</sup>Significance of a Wilcoxon rank-sum test after Benjamini-Hochberg correction. Bold text indicates P values < 0.001.
Table II. Correlation coefficients of the overrepresentation analysis of functional annotations among genes that are coexpressed with REIL2 under cold control or under temperature control

| Data Set | Cold-Controlled Coexpression | Temperature-Controlled Coexpression |
|----------|-----------------------------|-------------------------------------|
| Bin      | Bin Name                    | Bin Size   | Caldana et al. (2011) | Kaplan et al. (2007) | Kilian et al. (2007) | Caldana et al. (2011) | Kaplan et al. (2007) | Kilian et al. (2007) |
| Negative coexpression | 1 | PS | 187 | -0.257 | -0.145 | -0.370 | -0.168 | -0.140 | -0.155 |
| 1.1 | PS.lightreaction | 136 | -0.324 | -0.130 | -0.311 | -0.302 | -0.144 | -0.101 |
| 11 | lipid metabolism | 375 | -0.100 | -0.092 | -0.119 | -0.082 | -0.099 | -0.088 |
| 27.3.5 | RNA.regulation of transcription. ARR | 23 | -0.558 | -0.327 | -0.347 | -0.106 | -0.164 | -0.197 |
| 27.3.6 | RNA.regulation of transcription. bHLH, Basic Helix-Loop-Helix family | 93 | -0.257 | -0.188 | -0.243 | -0.163 | -0.112 | -0.123 |
| Positive coexpression | 27.1 | RNA.processing | 254 | 0.110 | 0.181 | 0.163 | 0.033 | 0.127 | 0.186 |
| 27.1.2 | RNA.processing,RNA helicase | 29 | 0.261 | 0.398 | 0.285 | 0.222 | 0.335 | 0.198 |
| 29.2 | protein.synthesis | 515 | 0.132 | 0.200 | 0.175 | 0.194 | 0.260 | 0.194 |
| 29.2.1 | protein.synthesis.ribosomal protein.eukaryotic.40S subunit | 371 | 0.085 | 0.164 | 0.126 | 0.158 | 0.254 | 0.173 |
| 29.2.1.2 | protein.synthesis.ribosomal protein.eukaryotic.60S subunit | 236 | 0.202 | 0.294 | 0.333 | 0.329 | 0.433 | 0.307 |
| 29.2.1.2.1 | protein.synthesis.ribosomal protein.eukaryotic.40S subunit | 88 | 0.185 | 0.272 | 0.302 | 0.295 | 0.413 | 0.291 |
| 29.2.1.2.2 | protein.synthesis.ribosomal protein.eukaryotic.60S subunit | 148 | 0.212 | 0.307 | 0.352 | 0.349 | 0.445 | 0.316 |
| 29.2.2.50 | protein.synthesis.misc ribosomal protein.BRIX | 6 | 0.733 | 0.723 | 0.874 | 0.731 | 0.818 | 0.826 |
| 29.2.3 | protein.synthesis.initiation | 84 | 0.282 | 0.356 | 0.392 | 0.299 | 0.311 | 0.278 |

*Average Spearman’s rank correlation to REIL2 of each reported bin. Bold text indicates P values < 0.001.

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positively correlated, except for the negatively correlated stress-seventy subfamily A gene1 (ssa1) and ssa2 and the noncorrelated rehl1 gene.

The equivalent *A. thaliana* coexpression matrices were highly similar to the yeast matrices (Fig. 7; Supplemental Fig. S10). Only the coexpression of one of the two *A. thaliana* nucleolar G-protein gene1 paralogs, namely At1g10300, and four of the six ssa1/ssa2 paralogs, At5g02500, At5g02490, At3g09440, and At1g56410, did not match to their yeast homologs. As judged by the average correlation coefficients, the *A. thaliana* genes were less tightly intercorrelated than the yeast genes.

The coexpression pattern of *A. thaliana* REIL2 and REIL1 was similar to the patterns of reil and rehl in yeast (Fig. 7). In yeast, reil was strongly coexpressed, whereas rehl was not. In *A. thaliana*, REIL1 correlation was attenuated compared with REIL2 and did not pass the stringent criteria of our preceding coexpression analysis. This was expected according to our initial test, which showed the absence of a significant temperature response of REIL1 transcription.

**REIL1 Complements the Growth Defect of the Yeast Δreil Deletion Mutant**

To test complementation, we established a temperature-controlled, liquid culture, microtiter plate reader assay set to 28°C and 22°C. The Δreil deletion mutant did not differ significantly from the BY4742 (S288C) wild type in regard to cell size and cell density of exponentially grown cultures adjusted to an optical density at 595 nm (OD595) of 1.0. The respective populations of wild-type cells had 1.4 ± 0.1 107 cells mL−1 and an average cell size of 4.9 ± 0.3 μm. The Δreil deletion mutant had a cell density of 1.2 ± 0.2 107 cells mL−1 and 5.1 ± 0.4 μm average size. As a consequence, we recorded OD595 as a comparable measure for mutant and wild-type growth. We tested the lag phase and the maximal growth rate from high-resolution growth kinetics as potential indicators for complementation of the Δreil growth deficit by heterologous expression of the full-length REIL1 or REIL2, compared with homologous expression of yeast Reil1p (Supplemental Fig. S11). The yeast Δreil deletion mutant (Iwase and Toh-e, 2004; Lebreton et al., 2006) and the wild type did not differ in regard to the lag phase. The lag phases of the wild type and the mutant were approximately 78 ± 26 min at 28°C and almost doubled to 150 ± 24 min at 22°C (Fig. 8). By contrast, the maximum relative growth rate of the Δreil deletion mutant was significantly reduced 0.72-fold compared with the wild type at both temperatures. Upon prolonged cultivation at 22°C or 28°C, the Δreil deletion mutant reached identical or even slightly higher
cell densities than the wild type. The wild type entered a death phase following the stationary phase. By contrast, the \( D_{\text{rei1}} \) mutant still increased OD\( _{595} \), however, at a low rate (Supplemental Fig. S11). This phenomenon was not reversed to the wild type by expression of yeast \( \text{Rei1p} \) and rendered simple colony complementation assays on solid media difficult to score. In conclusion, the maximum relative growth rate in liquid culture was the best available measure to test complementation of the \( D_{\text{rei1}} \) mutant phenotype.

The expression of yeast \( \text{Rei1p} \) and of \( \text{Rei1} \) but not the expression of \( \text{Rei1} \) complemented robustly the \( \Delta \text{rei1} \) growth defect at suboptimal temperatures (Fig. 8). In detail, expression of \( \text{REIL1} \) restored on average 92% of the wild-type maximum relative growth rate at 28°C and 85% at 22°C. Heterologous expression of \( \text{REIL1} \) was, thus, almost as efficient compared with the approximately full complementation by expression of the endogenous yeast \( \text{Rei1p} \).

**DISCUSSION**

The Link of \( A. \text{thaliana} \) REIL Proteins to Cold Stress Physiology

To cope with cold stress, \( A. \text{thaliana} \) can cold acclimate. Cold acclimation activates metabolic and transcriptional changes (Hannah et al., 2005; Kaplan et al., 2007; Guy et al., 2008), which allow leaves that were generated under optimal temperature conditions to survive and successfully function in suboptimal temperature conditions. Much is known about the molecular basis and transcriptional regulation of plant cold acclimation (Fujita et al., 2006; Thomashow, 2010), while the developmental programming that leads to the production of new leaves with a specific cold morphology only recently moved into the focus of attention (Gorsuch et al., 2010a, 2010b).

We present evidence that the \( A. \text{thaliana} \) REIL paralogs are required at two checkpoints of leaf development in the cold. First, the \( \text{rei1-1 rei2-1} \) double mutant arrests development in the cold prior to the cell proliferation of the nascent leaf (Figs. 2 and 3). The presence of either \( \text{REIL1} \) or \( \text{REIL2} \) is required for cell proliferation in the cold but not at optimal temperature. The developmental arrest of the \( \text{rei1-1 rei2-1} \) double mutant appears to be similar to the growth arrest of the \( \text{bps1-2} \) mutant (Van Norman et al., 2011). The \( \text{bps1-2} \) mutant arrests at 16°C, also prior to the emergence of the first rosette leaves. The function of \( \text{Bypass} \), however, appears to be linked to a still unknown root-derived signal, which is required for general leaf development. Other than the \( \text{rei1-1 rei2-1} \) double mutant, \( \text{bps1-2} \) is affected both at suboptimal and at optimal growth temperatures.

Second, the allelic \( \text{reil2} \) mutants indicate the existence of a subsequent developmental checkpoint in the cold during early leaf development (Figs. 3 and 4). \( \text{REIL2} \) but not \( \text{REIL1} \) is required to pass this checkpoint and to maintain normal leaf growth in the cold. The loss of \( \text{REIL2} \) function causes small, spoon-shaped leaves and an arrest of leaf growth. \( \text{REIL2} \) is only required during vegetative growth in the cold. A so-far unknown signal, which coincides with the appearance of inflorescences in the cold (Fig. 5), overrides the \( \text{REIL2} \)-mediated arrest or allows \( \text{REIL1} \) to take over the function of \( \text{REIL2} \). A similar phenomenon appears to be associated with a shift to optimal temperatures (Figs. 4 and 5).

The Conservation of \( \text{REIL} \) Function in \( A. \text{thaliana} \)

The \( A. \text{thaliana} \) REIL proteins are a striking example of functional conservation between plant and yeast orthologs. Two paralogs with high sequence homology exist in each species. Loss of one paralog, namely yeast \( \text{Rei1p} \) or \( A. \text{thaliana} \) \( \text{REIL2} \), causes a conditional growth defect in the cold. Loss of the second paralog alone appears to be irrelevant for the cold response. But the loss of both paralogs shows that \( \text{REIL} \) proteins...
are essential for the growth at even slightly suboptimal temperatures.

We currently interpret these matching observations as a case of conserved function. We are well aware of the fact that a growth defect of a unicellular organism, such as yeast, and a biphasic arrest of the development and growth of the multicellular leaf organ will likely share basic aspects but will also certainly involve fundamentally different mechanistic details. We also caution that the conserved functional context may not necessarily be the result of a phylogenetic conservation. As we currently lack evidence from other species, the conserved function may also result from convergent evolution. The main indicators of convergence are the clearly independent gene duplications in the course of yeast and A. thaliana phylogeny (Fig. 1B; Supplemental Fig. S3).
The Association between A. thaliana REIL Proteins and Eukaryotic Ribosomal Maturation

In this initial study on the A. thaliana REIL proteins we decided to explore those molecular aspects that are shared between A. thaliana and yeast. So far, the annotation of A. thaliana REIL proteins was based on the presence of zinc finger domains. A. thaliana REIL proteins may have other functions than the initially inferred transcriptional regulation, not least because the small fraction of evolutionary conserved zinc finger proteins to which Rei1p, Rei1p, and the A. thaliana REIL proteins belong comprise a high number of RNA-interacting proteins (Engbrecht et al., 2004).

A first link of A. thaliana REIL proteins to ribosomal function was indicated by the pointed-leaves phenotype and the slight growth retardation of the rei1-1 rei2-1 double mutant at optimal temperature. The first described pointed-leaves mutation pf1 was an allele of the ribosomal RIBOSOMAL PROTEIN OF THE SMALL SUBUNIT18A (RPS18A) gene. The pf1 mutation changes the shape of the lamina of juvenile leaves to be pointed (Van Lijsebettens et al., 1994). Subsequently, mutations of several other structural ribosomal genes, e.g., RPS5A, RPS13A, RPS21B, RPS24B, RPL4D, RPL5A, RPL5B, RPL7B, RPL10aB, RPL18C, RPL28A, and RPL39C, were found to cause similar developmental and growth phenotypes. The high number ribosomal genes causing similar effects clearly indicate a link between eukaryotic ribosomes and leaf development (Horiguchi et al., 2011).

Because the yeast Rei1p also had a well-described role in eukaryotic ribosomal maturation, we focused on testing the A. thaliana REIL proteins for functional conservation. Our coexpression analysis led us from the proof of enrichment of the functional categories, translation initiation, and protein synthesis among the coexpressed genes (Tables I and II) to the discovery that REIL2 and rei1 are specifically coexpressed with genes that transiently associate with eukaryotic ribosomes at all main steps of ribosomal maturation (Supplemental Table S3). The genes with conserved coexpression were linked predominantly to the maturation machinery of eukaryotic ribosomes or to structural ribosomal genes, namely RPS2, RPS4, RPL27, and RPL35A (Supplemental Table S3). The list contained six 90S preribosome proteins, U three protein7 (Utp7p), Ribosomal RNA processing protein9 (Rrp9p), Pre-mRNA processing protein4, Nucleolar protein9, Nucleolar complex-associated protein4 (Noc4p), and Utp15p. Specifically, Utp7p is part of the U3 protein complex required for transcription 90S preribosomal assembly complex (Henras et al., 2008). Rrp9p belongs to the U3 small nucleolar ribonucleoprotein. U3 small nucleolar ribonucleoprotein is, next to the t-UTP complex, a second particle required for 90S assembly (Henras et al., 2008). Four other members of the short list, namely Core interacting component1, Nuclear import protein7, Suppressor of ste4 protein1, and Suppressor of ste4 protein2, are associated with the 66S preribosomal subunit that is downstream of the 90S particle and precedes the nuclear pre-60S subunit (Horsey et al., 2004). Furthermore, Utp14p and Utp23p and the previously mentioned Rrp9p, Noc4p, Utp7p, and Utp15p are part of the 40S processome that precedes the nuclear pre-40S particle (Dragin et al., 2002). Noc4p is, in addition, part of the 40S preribosome export complex (Henras et al., 2008). Noc2p belongs to the Noe1p-Noc2p and Noc3p-Noc2p complexes that are required for the intranuclear trafficking of the preribosomes (Milke et al., 2001). Ribosome assembly protein4 and Severe depolymerization of actin protein1 of the short list both need to be removed from the pre-60S particle prior to export into the cytosol. The absence of these proteins causes nuclear retention of the pre-60S particles (Henras et al., 2008; Panse and Johnson, 2010). We would like to highlight the fact that REIL2 is coexpressed with the Nmd3 and...
Tif6 orthologs At2g03820 and At3g55620. The Nmd3p and Tif6p of yeast are specifically involved in the final steps of the cytosolic ribosome maturation process. Nmd3p is required for nuclear export of the 60S pre-ribosome and one of the last factors to be released in the cytosol before the 60S subunit becomes translationally active. Nmd3p is also discussed to perform structural proofreading, a process that is thought to ensure that only correctly assembled subunits are exported from the nucleus and rendered active (Johnson et al., 2002; Lo et al., 2010). Tif6p acts as an antisassociation factor for the yeast ribosome as was recently shown by crystallography (Gartmann et al., 2010; Klinge et al., 2011). As a consequence, Tif6p needs to be released from the pre-60S subunit during the final steps of cytosolic maturation. Based on this knowledge, we finally made the observation that the genes of the cytosolic ribosomal maturation machinery of yeast, including rei1, and of their A. thaliana orthologs, including REIL1 (Fig. 6), have highly similar coexpression matrices (Fig. 7; Supplemental Table S4).

To further support the so-far circumstantial evidence of our coexpression analysis, which indicated that A. thaliana REIL proteins may be essential for ribosomal maturation in the cold, we initially considered tests for changes of the composition of ribosomal RNAs and respective precursors. We so-far rejected this approach because mutations that impair eukaryotic ribosome biogenesis in yeast typically do not lead to a massive accumulation of the corresponding pre-ribosomal RNAs (Henras et al., 2008). Therefore, respective observations in A. thaliana will be difficult to interpret and compare. Instead, we demonstrated functional complementation of the ∆rei1 mutant growth phenotype by REIL1, which is the strongest evidence so far for functional conservation. Under the conditions currently tested, A. thaliana REIL1 can still almost completely substitute yeast Rei1p function. By contrast, REIL2 appears to be mutated beyond full complementation, a fact to be expected of an isoform that plays a plant-specific role in leaf development at low temperatures.

CONCLUSION

We demonstrate that A. thaliana activates a conditional developmental program that is required for leaf development and growth in the cold. This program involves REIL proteins that are conserved parts of the maturation machinery of eukaryotic ribosomes. We see our study as a first step toward a deeper understanding as to how a housekeeping process such as eukaryotic ribosomal maturation may be linked to the developmental processes of plant cell differentiation or leaf organ growth and as to how this process may be modified by low temperatures. Even mildly lowered temperatures inherently slow down biological processes. Our germination assays, for example, clearly demonstrate that the speed of germination is about halved by a temperature shift from 20°C to 10°C. Germination is even further delayed when one or both REIL proteins are missing. The deceleration of biological processes is generally explained by the cold-induced reduction of enzyme activities. But those processes that involve the formation or the dissociation of protein complexes, such as the assembly of a ribosome, will also be slower. We currently think that the release of maturation factors, such as Nmd3p and Tif6p, may be severely delayed by low temperatures and that the role of REIL proteins may be the facilitation of these dissociation processes at low temperatures.

Our investigations of REIL function are ongoing and will shed new light on the relationship between housekeeping genes and plant development, an interaction that was found to be more important and widespread than previously thought (Tsukaya et al., 2013). We hope to contribute novel aspects to the recently reviewed and systematically investigated interaction of the plant ribosome with leaf development (Schippers and Mueller-Roeber, 2010; Horiguchi et al., 2011, 2012).

MATERIALS AND METHODS

Identification of T-DNA Insertion Mutants of REIL1 and REIL2

Seeds of the T-DNA insertion mutants SALK_090487 (rei1-1) and SALK_040068 (rei2-2) were obtained through the Nottingham European Arabidopsis Stock Centre (Scholl et al., 2000). The T-DNA mutant GK_166C10 (rei2-1) was generated through the GABI-Kat program (Rosso et al., 2003) and was provided by Bernd Weisshaar (Max Planck Institute for Plant Breeding Research). Homozygous lines were generated in this study and confirmed by PCR amplification of genomic DNA with rei1 gene-specific and T-DNA-specific oligonucleotides (Supplemental Table S5). PCR was performed using 36 cycles of 15 s denaturation at 94°C followed by 30 s annealing at 55°C and 2 min elongation at 72°C. The T-DNA-specific oligonucleotides were outward-directed left-border primers LBb1 (SALK) and GKBs409 (GABI-Kat). The REIL2 (At2g24500) and REIL1 (At4g314200) gene-specific primers were positioned 5′ and 3′ relative to the approximate T-DNA insertion site reported by the seed stock providers. The 5′-oligonucleotide primers at2g24500for and at4g314200for were positioned at the stop codons, and the 3′-oligonucleotide primers at2g24500rev and at4g314200rev were placed at the stop codons. The exact T-DNA insertion sites of each line were determined by sequencing of the left-border insertion site. In agreement with the genetic background of the mutants, Col-0 was used as wild-type control throughout this study.

Total RNA for reverse transcription PCR (Supplemental Fig. S4) was extracted with the RNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instructions. The complementary DNA (cDNA) was amplified from total RNA using oligo (dT)12-18 primers (Invitrogen Life Technologies) and the SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). The PCR reactions were performed with the primer pairs at2g24500for and at2g24500rev (REIL2) and at4g314200for and at4g314200rev (REIL1). The actin2 transcript was amplified by primers act2_for and act2_rev (Supplemental Table S5).

Sequence Analysis and Comparison Tools

Sequence searches and analyses were performed using the National Center for Biotechnology Information protein and nucleotide sequence repository, the National Center for Biotechnology Information BLAST resources (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997), and the Bioinformatics Toolkit (http://toolkit.tuebingen.mpg.de; Bieger et al., 2006). Protein sequences were either taken from BLASTp results or manually translated from retrieved complementary DNA or genomic sequences. Phylogenetic conservation analysis between Arabidopsis thaliana and yeast (Saccharomyces cerevisiae) was performed using the InParanoid (Version 7) annotations of eukaryotic COGs (http://inparanoid.sbc.su.se/cgi-bin/index.cgi; O’Brien et al., 2005; Osiuld
et al., 2010). Multiple sequence alignments for phylogenetic tree analysis were generated using ClustalX (Thompson et al., 1997) with gap-opening penalty set to 15 and gap extension set to 0.3. PHYLIP version 3.68 (http://evolution.genetics.washington.edu/phylip.html) was applied for inference of phylogenetic trees using the maximum-likelihood method, bootstrapping, and the display of a rooted version of the resulting consensus tree (Felsenstein, 1989).

Seed Batches, Germination, and in Vitro Growth Assays

At least two independent seed batches of each homozygous genotype were generated using plant populations that completed a full life cycle from germination to seed setting and harvest under optimum temperature conditions. All seed batches were stored at 4°C for at least 1 month prior to the assessment of germination and growth behavior. Seeds were sterilized at room temperature with sodium hypochlorite and germinated without additional stratification using one-half-strength Murashige and Skoog-Agar plates, 0.8% (w/v) agar, and 2% (w/v) Suc (Murashige and Skoog, 1962). Seeds for germination assays were plated equally spaced in batches of approximately 80 per mutant line or the Col-0 wild type. The assays were performed in controlled-environment chambers under sterile conditions with a transparent lid. Germination assays were performed in short days with an 8-h/16-h day/night cycle at 20°C/16°C and 100 to 150 μmol m⁻² s⁻¹ light intensity using a controlled-environment chamber. After 2 weeks, the plantlets were transferred at stage approximately 1.02 to 1.03 into pots of 10-cm diameter soil (Stender AG). Acclimation to soil was in short days with an 8-h/16-h day/night cycle at 20°C/16°C optimum temperature conditions. All experiments used complete rosettes of nonacclimated plants and were controlled by diurnal time series at 20°C to 21°C optimum temperature. From these studies, we selected only the experiments that were performed in parallel using a Latin square cultivation array. Number of rosette leaves greater than 1 mm, planar rosette leaf area (mm²), convex hull of the rosette (mm²), and surface coverage (%) of the plant rosettes were determined manually supervised using Adobe Photoshop CS4 Extended (Version 11.0) and normalized to the scale within each photograph. We followed previous recommendations for automated morphometric analyses of the A. thaliana rosette (Jansen et al., 2009; Walter et al., 2009), where the surface coverage of a rosette is defined as the proportion (%) of planar leaf area inside the rosette's convex hull and where the convex hull is defined as the area inside the shortest line around a plant rosette.

Gene Annotations and Functional Overrepresentation Analysis

Yeast gene names, gene identifiers, and functional annotations were from the Saccharomyces Genome Database (http://www.yeastgenome.org). Yeast orthologs were reported by the InParanoid 7 resource. Most yeast orthologs reported by the InParanoid 7 resource were among these pairs of A. thaliana and yeast orthologs reported by the InParanoid 7 resource. The human MATE FAMILY TRANSPORTER (MATE), MYOELASTOSIS FAMILY TRANSPORTER (MATE), PATR familial protein (PAT), and yeast orthologs reported by the InParanoid 7 resource were according to The Arabidopsis Information Resource (http://www.arabidopsis.org) with functional classifications obtained from MapMan (Usadel et al., 2005). Overrepresentation analysis using the MapMan functional bin annotations and Benjamini-Hochberg-corrected Wilcoxon rank-sum significance was as described previously (Usadel et al., 2005). Robustly overrepresented functional categories were obtained by intersection applying a common P < 0.05 threshold to independently generated and processed transcript data sets (compare with below).

Analysis of COGs

Phylogenetic conservation was judged according to the InParanoid 7 resource on conserved genes (O’Brien et al., 2005; Ostlund et al., 2010). This database annotated 8,046 pairs of orthologs between yeast and A. thaliana that were grouped into 2,045 COGs. Seven thousand two hundred eight orthologous gene pairs were present in the selected yeast and A. thaliana transcript studies. We thus compared the REL1 coexpression with 5,026 A. thaliana genes to the rel1 coexpression with their respective 2,378 phylogenetically conserved yeast orthologs. Homologs of the human MATERNAL EMBRYONIC LEUCINE ZIPPER KINASE (MEK), MYOELASTOSIS FAMILY TRANSPORTER (MATE), PATR familial protein (PAT), and yeast orthologs reported by the InParanoid 7 resource were according to The Arabidopsis Information Resource (http://www.arabidopsis.org) with functional classifications obtained from MapMan (Usadel et al., 2005). Three studies comprising independent time series analyses following a cold response (Bieniawska et al., 2008) and general transcriptome analyses were performed in parallel using a Latin square cultivation array. Number of rosette leaves greater than 1 mm, planar rosette leaf area (mm²), convex hull of the rosette (mm²), and surface coverage (%) of the plant rosettes were determined manually supervised using Adobe Photoshop CS4 Extended (Version 11.0) and normalized to the scale within each photograph. We followed previous recommendations for automated morphometric analyses of the A. thaliana rosette (Jansen et al., 2009; Walter et al., 2009), where the surface coverage of a rosette is defined as the proportion (%) of planar leaf area inside the rosette's convex hull and where the convex hull is defined as the area inside the shortest line around a plant rosette.

Gene Expression Data Sets

Gene expression studies for the analysis of cold- and temperature-controlled coexpression modules were selected according to a best match of the respective experimental descriptions. Genome-wide time series analyses to cold-stress cues were searched, starting with previous comparative reports on the A. thaliana cold response (Bieniawska et al., 2008) and general transcriptome archiving resources (Zimmermann et al., 2004; Hruz et al., 2008). Three studies that investigated the transcriptional response of REL1 to cold and heat in illuminated rosette leaves under similar conditions were selected for coexpression analysis (Kaplan et al., 2007; Kilian et al., 2007; Caldana et al., 2011). These three studies comprised independent time series analyses following a temperature shift from optimal temperature to 4°C or to 32°C to 42°C heat. From these studies, we selected only the experiments that were performed in the light. All experiments used complete rosettes of nonacclimated plants and were controlled by diurnal time series at 20°C to 21°C optimum temperature. Equivalent transcriptome profiling experiments for the comparative coexpression analysis of yeast, were previously performed and reported by our...
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laboratory (Strasburg et al., 2010; Walther et al., 2010). We selected two similar transcriptome analyses for robustness analysis (Gasch et al., 2000; Sahara et al., 2002).

The analysis of the transcriptional responses of REIL1 and REIL2 to a wide range of abiotic stresses was initiated with a single reference data set (Kilian et al., 2007), confirmed and complemented by independent experiments that were archived by the Genevestigator database for the meta-analysis of transcriptional profiles (Zimmermann et al., 2004; Hruz et al., 2008).

Comparative Coexpression Analysis

Only REIL2 was subjected to a systematic gene-targeted coexpression analysis. REIL1 transcript levels were not significantly changed under cold or heat stress and therefore not deemed suitable for a correlation-based coexpression analysis under the influence of these stress factors. The normalized, preprocessed, and corrected transcriptome data published by each selected study were used. All time series data selected for this study were corrected by subtraction of logarithmic expression values of the respective temporal controls prior to coexpression analysis. This procedure was necessary to account for the known diurnal effects that are active in a photoautotrophic plant (Bieniawska et al., 2008). We applied the same correction procedure to the yeast gene expression data, which were profiled in batch culture.

The A. thaliana transcriptome data sets were (1) the rosette leaf data from the cold and heat subset of a global stress response compendium (Kilian et al., 2007), (2) the data of a microtiter plate experiment, (2) the data of a study on the cold stress response of A. thaliana rosettes with paralleled heat stress data submitted to the NASCAarrays (Kaplan et al., 2007), and (3) part of the data of a combinatorial stress study on the interactions of temperature and light regimes in A. thaliana rosettes (Caldana et al., 2011). Of the latter, only the subset of illuminated samples was used. The 4°C cold stress data obtained at 85 μEm⁻² sec⁻¹ were corrected by the 21°C and 75 μEm⁻² sec⁻¹ set. The 32°C and 150 μEm⁻² sec⁻¹ heat stress data were corrected using the also available 21°C set at identical light intensity. The transcriptome data of the budding yeast, were the previously published time series analyses following heat or cold stress performed by our laboratory (Strasburg et al., 2010; Walther et al., 2010) and combined from previously published studies on cold stress (Sahara et al., 2002) and a compendium of abiotic environmental stress responses (Gasch et al., 2000). Of the latter data set, we used the temperature shift from 37°C to 25°C and the continuous growth at various temperatures to complement the five time points after cold stress of Sahara et al. (2002) for the cold-controlled coexpression analysis. The general temperature control analysis was performed together with the 37°C heat shock from 25°C, the 37°C heat shock from various temperatures, and the mild 33°C heat shock experiments of Gasch et al. (2000). Spearman rank correlations were calculated as a robust outlier-insensitive measure for the coexpression analysis (Steinhauer et al., 2004). The correlation coefficient was used throughout this study. This measure allowed the ranking of A. thaliana and yeast genes according to best positive, +1.0, or best negative, −1.0, coexpression with REIL2 and reil1, respectively. The transcriptome data sets were independently analyzed to allow the averaging across experiments or a more stringent robustness testing by intersection analysis (Sanchez et al., 2010).

Yeast Complementation Analysis

The yeast Δreil1 mutant, genotype BY4742, Mat a, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, YBR267wΔ1-tamMX4, and the parent strain BY4742, Mat a, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, derived from yeast SK288C (Winzeiler et al., 1999) were obtained from EUROSCARF. Full-length complementary DNAs of reil1, REIL1, and REIL2 were cloned into the low-copy Gateway (Alberti et al., 2007) destination vector pAG305GPD-ccdB (Addgene plasmid 14154) and transformed into the yeast Δreil1 mutant. Positive transformed colonies were screened by complementation of the Leu autotrophy. The presence of the correct plasmid was confirmed by PCR. The presence of the Δreil1 mutant background was confirmed by resistance to 0.05 mg mL⁻¹ kanamycin. The average cell size and cell density of cultures taken during exponential growth and adjusted to OD595 of 1.0 were analyzed by a Cellometer Auto M10 system (Nexcelom Bioscience) according to the manufacturer’s instructions. The growth assays were performed using a temperature-controlled, automated microtitter plate reader system that was set to 28°C and 22°C, respectively. Lower operating temperatures were beyond system specifications. Yeast strains were precultured in liquid yeast peptone dextrose medium. All cultures were adjusted carefully prior to the growth assay to OD595 of 0.4 for the 28°C assay or to OD595 of 0.8 for the assay at 22°C. Kanamycin was omitted so as to allow the comparison to both the Δreil1 gene deletion mutant and the nonresistant wild-type genotype BY4742. Four microtitter plates with eight 200-μL cultures of each strain were assayed independently at each temperature. In total, the growth curves of 32 cultures of each yeast strain (eight replicates per plate) were recorded at 20-min intervals over 24-h periods (Supplemental Fig. S11). The natural logarithm (ln) of Nt/N0 was plotted as a function of time, where Nt is the initial OD595 of each culture and N0 is the OD595 of the same culture at time t, assuming OD595 to be proportional to cell density (N). The maximum relative growth rate (μmax) was defined as the slope of the tangent at the inflection point of the semilogarithmic growth curve. The inflection point of this growth curve was found at the maximum of the first derivative function. The tangent was defined as the linear function \( \ln(N_t/N_0) = \mu_{max}t + b \), where t is the time and b the y-axis intercept. The log phase (λ) was defined as the y-axis intercept of this tangent and was calculated by λ = −b/μ, where \( \ln(N_t/N_0) = 0 \). The unit of λ is min⁻¹. The unit of μmax was min⁻¹. Note that μmax and λ were calculated from the initial phases of the growth assays (Supplemental Fig. S11), avoiding the influence of the transition phase to stationary phase. The asymptote of the growth curve representing maximal cell density was not analyzed because of an unusual behavior of the Δreil1 mutant in stationary phase. Significance of changes compared with the BY4742 wild type or compared with the Δreil1 mutant was judged by Student’s t test. The complementation result was confirmed using the integrating destination vector pAG305GPD-ccdB (data not shown). Furthermore, we confirmed restoration of growth in the presence of kanamycin omitting the BY4742 wild type using only the Δreil1 mutant and the respective transformants with the pAG305GPD-ccdB constructs (data not shown).

Sequence data of this study were retrieved from The Arabidopsis Information Resource (http://www.arabidopsis.org/index.jsp), the Yeast Genome Database (http://www.yeastgenome.org/), or GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under the following accession numbers: REIL1 (AAG31420), REIL2 (A024900), reil1 (YLR387C), ZFRP1 (YNL652W), MELK (Y14680), B-MYB (YTH034), and ASK1 (Y99663). The accession numbers, taxonomy, and protein codes of all Reil1p-like homologs investigated in the phylogenetic analysis of this study are listed in Supplemental Table S1. The accession numbers of yeast and A. thaliana genes analyzed in the coexpression study are listed in Supplemental Table S3. Note that we applied the yeast convention to report yeast proteins, genes, and mutants, e.g. Rei1p, reil1, and Δreil1, respectively.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Genomic and mRNA sequence alignment of the A. thaliana REIL genes and of orthologs from A. lyrata, T. halophilus, and B. rapa ssp. pekinensis.

Supplemental Figure S2. Alignment of the REIL proteins of A. thaliana, A. lyrata ssp. lyrata, T. halophilus, and B. rapa ssp. pekinensis.

Supplemental Figure S3. Phylogenetic analysis of eukaryotic Reil1p-like orthologs.

Supplemental Figure S4. Reverse transcription PCR test for the absence of full-length transcripts from rosette leaves of the reil1-1, reil1-2, and reil2-2 mutants before (+flower) and after (+flower) appearance of flower buds at 10°C and at 20°C.

Supplemental Figure S5. Examples of the differential temperature control of reil1 and reil1 gene expression in yeast (Strasburg et al., 2010) compared with the temperature response of REIL1 and REIL2 transcript levels in A. thaliana (Caldana et al., 2011).

Supplemental Figure S6. Root length of reil mutants and the Col-0 wild type determined under control conditions using a vertical plate in vitro assay.

Supplemental Figure S7. Sensitivity of reil mutants and the Col-0 wild type to stress factors that are listed within the inserts of this figure.

Supplemental Figure S8. The gene sets that are positively coexpressed with REIL2 are enriched with genes that are conserved between A. thaliana and yeast.

Supplemental Figure S9. Comparative coexpression analysis of A. thaliana genes to REIL2 with the coexpression of their respective yeast orthologs with yeast reil1.
Supplemental Figure S10. Comparison of the coexpression matrix of the yeast 60S cytoplasmic maturation machinery to the coexpression matrix of A. thaliana paralogs.

Supplemental Figure S11. Representative growth curves, semilogarithmic plots, where the cell density is assumed to be proportional to OD595, and first derivative plot of the semilogarithmic growth curve.

Supplemental Table S1. List of eukaryotic reil-like orthologs listed according to the abbreviations used in Supplemental Figure S3.

Supplemental Table S2. Germination rates of the reil single gene and double mutant lines compared with the Col-0 wild type at 20°C.

Supplemental Table S3. Table of A. thaliana genes that show a robust coexpression with RELIL2 and a conserved coexpression of their yeast orthologs with reil.

Supplemental Table S4. Coexpression table of the A. thaliana genes and their yeast paralogs, which are known to be functionally involved in the cytoplasmic maturation of the eukaryotic 60S large ribosomal subunit (compare with Fig. 6).

Supplemental Table S5. List of oligonucleotide primers used to characterize the reiL-1 (SALK_004861), reiL-2 (GK_166110), and reiL-2 (SALK_040068) T-DNA insertion mutants.

Supplemental Document S1. List of detailed legends to Supplemental Figures S1–S11 and Supplemental Tables S1–S5.

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