Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11433–11437, December 1993
Plant Biology

The *Arabidopsis* endoplasmic reticulum retention receptor functions in yeast

(KDEL receptor/*ERD2* gene/yeast complementation/*Arabidopsis* cDNA library)

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Communicated by Anton Lang, September 3, 1993 (received for review, August 9, 1993)

ABSTRACT **Soluble proteins retained in the lumen of the endoplasmic reticulum (ER) contain a carboxyl-terminal tetrapeptide sequence that functions presumably to recycle these proteins from a subsequent compartment. Biochemical and genetic evidence indicate that the *ERD2* gene product is the receptor for these ER retention signals. Here we report the identification of a cDNA clone from *Arabidopsis thaliana* (*aERD2*) similar in sequence and size to members of the *ERD2* gene family. Southern and Northern blot analyses indicate that *Arabidopsis* contains a single *aERD2* gene which is expressed at different levels in various plant tissues. A functional assay demonstrates that the *Arabidopsis* homologue, unlike the mammalian protein, can complement the lethal phenotype of the *erd2* deletion mutant of *Saccharomyces cerevisiae*, indicating that this protein may have a similar function in plants. As the plant protein may have a binding specificity similar to the human Erd2 protein but can function in yeast, we suggest that the plant homologue is the functional link between yeast and animals.**

Entry of proteins into the endomembrane system of eukaryotic cells generally requires an amino-terminal signal peptide that facilitates translocation into the endoplasmic reticulum (ER) (1). Positive signals are required for retention by the cells; proteins lacking a signal are secreted in all systems tested (2–4). Targeting signals for delivery of proteins to lysosomes and vacuoles of animals, plants, and yeast have been identified (5–7) and appear to be different in all three systems (8). Retention sequences of membrane proteins in ER and Golgi have also been studied (9–12). However, among the best characterized endomembrane retention signals are the carboxyl-terminal tetrapeptide sequences of soluble proteins which reside in the lumen of the ER. These sequences are typically K/H/RDEL in mammals (4, 13), H/DDEL in yeasts (14, 15), and K/H/RDEL in plants (16, 17). Previous work has shown that these carboxyl-terminal sequences are necessary for retention of soluble proteins in the lumen of the ER (4, 13, 16), probably by continuous retrieval from a post-ER compartment (18). Although the identification of this compartment remains to be established it has been shown that some ER-resident proteins are modified by the addition of α1-6-mannose-linked residues, a characteristic of the early Golgi (19). In plants, there are examples of proteins which apparently contain a carboxyl-terminal KDEL sequence as deduced from their cDNA sequences (20–22) or artificially added to a storage protein (23) but are found in sites other than the ER. Although the final location of such proteins as the thiol proteases (21, 22) is not known, the auxin-binding protein is found at the plasma membrane (20) and chimeric phytohemagglutinin-KDEL localizes in the nuclear envelope and storage vacuoles (23). There are two possible explanations for this presumed "escape" from the ER retention machinery: either the KDEL is removed by a carboxypeptidase or the interaction between the ER retention receptor and the K/HDEL tetrapeptide is regulatable in plants.

A putative receptor for the ER-resident proteins was first identified as the *ERD2* gene product via a genetic approach in *Saccharomyces cerevisiae* (24). Subsequently, *ERD2*-homologous genes have been isolated by sequence homology from another yeast, *Kluyveromyces lactis* (25), and by polymerase chain reaction based on conserved regions from mammals (26–29). The mammalian protein has been localized by using epitope-tagged human Erd2 (26, 30) and antibodies to the carboxyl-terminal 21 amino acids of the bovine protein (29). In normal cells, the Erd2 protein is found in the Golgi, but the protein is redistributed to the ER when the cells also express high levels of an appropriate ligand, such as KDEL-tagged lysozyme (30). This supports the hypothesis that the Erd2 receptor recycles together with the ER protein back to that compartment. Recent *in vitro* binding studies with the human Erd2 protein show an intriguing pH-dependent binding curve which would also be consistent with such a hypothesis (31). This would then be similar to the pH-dependent binding of lysosomal proteins to the mannose 6-phosphate receptor (5) and may represent a basic concept about conditional binding of ligands and the principle of receptor recycling. The Erd2 receptor has two main functions, to bind the ER-retained proteins and to retrieve these proteins back to the ER. Recent work has begun to map the domains of the protein with respect to these functions (32). Despite 51% amino acid identity between the human and yeast proteins, the human *ERD2* homologue is unable to complement the loss of Erd2 function in yeast (26). Here, we describe the fortuitous cloning of the plant *ERD2* homologue from an *Arabidopsis thaliana* cDNA library and show that this gene is able to complement the yeast *erd2* mutant.*

MATERIALS AND METHODS

Materials. All chemicals used were of the highest purity commercially available. Restriction enzymes were from Boehringer Mannheim. *A. thaliana* plants of ecotypes RLD and Columbia were used. Plants grown in soil were kept in 16 hr of light and 8 hr of darkness, and plants in tissue culture were incubated under continuous light conditions.

***Arabidopsis* cDNA Library and Sequencing.** The *A. thaliana* cDNA library, λ-PRL2, is a λZipLox derivative containing *Sal* I–*Not* I cDNA inserts from ecotype Columbia wild type. The cDNA source was equal amounts of mRNA isolated from tissue culture-grown roots, 7-day-old etiolated seed-

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Abbreviations: ER, endoplasmic reticulum; FOA, 5-fluoroorotic acid.

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†The sequence reported in this paper has been deposited in the GenBank database (accession no. L23573).

lings, and rosettes and aerial tissue (stems, siliques, and flowers) from plants of different ages and two light regimes (continuous light or 16 hr light/8 hr dark). The amplified library comes from 2×10^5 primary transformants, and the size range of the inserts is 400–2000 bp. The library is available from the *Arabidopsis* Biological Resource Center at Ohio State University (Columbus, OH). Initially, the clones were sequenced with a Catalyst 800 (Applied Biosystems) using a T7 dye primer with *Taq* cycle sequencing chemistry. The products of the reaction were analyzed on a 373A sequenator (Applied Biosystems). The programs used to determine protein homology were of Gish and States (33) and Altschul *et al.* (34), available through the National Center for Biotechnology Information, and for hydropathy plots, that of Kyte and Doolittle (35). The complete sequence of the clone was obtained by using the method of Sanger *et al.* (36) and Sequenase from United States Biochemical.

DNA and RNA Isolation and Nucleic Acid Blots. *Arabidopsis* genomic DNA from ecotype RLD was isolated (37) from mature leaves from soil-grown plants; total RNA was prepared (38) from flower bud, stem, and leaf from soil-grown plants and roots from plants grown in liquid culture from *Arabidopsis* ecotype RLD. Southern and Northern blots were prepared (39) and probed with a ^{32}P-labeled *Cla* I–*Hin*dIII DNA fragment encompassing the coding region of the *Arabidopsis ERD2* (*aERD2*) gene made with [α-^{32}P]dATP (Amersham) by using random primers and Klenow DNA polymerase (Boehringer Mannheim).

Yeast Strains and Complementation of the *erd2* Mutant. The *S. cerevisiae* parental yeast strain, ΔLE26A, is a derivative of JCB102 (*MATα ade2 ade3 his3-Δ200 leu2*-3,122 *ura3-52 TRP1 erd2Δ*) described by Hardwick *et al.* (40). It carries a plasmid bearing the *TPI* promoter-driven *K. lactis ERD2* gene on a *CEN6* plasmid bearing *URA3* and *ADE3* and was kindly provided by H. R. B. Pelham (Cambridge, England). The *S. cerevisiae ERD2* gene in a *LEU2*-containing plasmid, pRSCErd2 (provided by H. R. B. Pelham), was replaced by the *Arabidopsis ERD2* gene in the sense or antisense orientation and each was transformed into the ΔLE26A strain by the LiCl method (41). Two independent colonies of each were tested on plates containing 5-fluoroorotic acid (FOA; ref. 42), and colonies growing on the FOA plates were tested for growth on minimal plates lacking leucine or uracil (43) compared with growth of the parental strain and the yeast transformed with the *Arabidopsis* gene in the antisense orientation.

RESULTS AND DISCUSSION

Isolation of cDNA Clone *aERD2* and Sequence Analysis of the Encoded Protein. It is likely that the plant Erd2 protein is structurally related to those previously isolated, because of the sequence conservation of *ERD2* between mammals and yeast (26, 27) and because of the sequence and antigenic similarity of the ER retention signals among plants, mammals, and yeast (15–17). However, despite extensive efforts, the *ERD2* homologue from plants has not been isolated by conventional cloning approaches, including polymerase chain reaction amplification. Partial sequencing of random clones from an *A. thaliana* cDNA library first identified a clone with significant homology to the *S. cerevisiae* Erd2 protein. Complete sequence analysis of this cDNA revealed that the clone was 970 bp long and contained 133 and 228 bp in the 5′ and 3′ untranslated regions, respectively (data not shown). Translation of the longest open reading frame revealed that it encoded a protein of 215 amino acids with a calculated molecular mass of 25.2 kDa, similar to the yeast and human Erd2 proteins (Fig. 1). The predicted amino acid sequence of the *Arabidopsis* homologue aligned with the *S. cerevisiae* (24) and human [erd2.1 (26)] Erd2 proteins is

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                .         .         .         .         .         .         60
A  MNIFRFAGDMSHLISVLILLLKIYATKSCAGISLKTQELYALVFLT-RYLDLFTDY-VSL
   :: ::  :: :::     :   :       ::: : : : : :: : ::::: :    ::
H  MNLFRFLGDLSHLLAIILLLLKIWKSRSCAGISGKSQVLFAVVF-TARYLDLFTNY-ISL
   :: ::  :: :::     :   :       ::: : : : : :: : ::::: :    ::
S  MNPFRILGDLSHLTSILILIHNIKTTRYIEGISFKTQTLYALVFIT-RYLDLLTFHWVSL

                .         .         .         .         .        120
A  YNSIMKIVFIASSLA-IVWCMRRHPLVR--RS----YDKDL--DTFRHQY-VVL-ACFVL
   ::  ::   :  :    :       :          :        :::          :   :
H  YNTCMKVVYIACSFTT-V-------LIYSKFKAT--YDGN--HDTFRVEFLVVPTA--IL
   ::  ::   :  :    :       :          :        :::          :   :
S  YNALMKIFFIV-STAYIVV-L----LQGSKRTNTIAYNEMLMHDTFKIQHLLIGSA---L

                .         .         .         *         .        180
A  GLILNE-KFTVQEVFWAFSIYLEAVAILPQLVLLQRS--GNVDNLTGQYVVF-LGAYRGL
          ::  :  : ::  :: :::::::     :  :     :  :  :  : :: :
H  AFLVNH-DFTPLEILWTFSIYLESVAILPQLFMV--SKTGEAETITSHYL-FALGVYRTL
          ::  :  : ::  :: :::::::     :  :     :  :  :  : :: :
S  MSVFFHHKFTFLELAWSFSVWLESVAILPQLYML--SKGGKTRSLTVHYI-FAMGLYRAL

                .         .         .         .        235
A  YIINWIYRYFTEDHFTRWIACVSGLVQTALYADFFYYYYISWKTNT-K-L-KLPA
   :  ::: ::  :      ::   :: :: :: :::: :  :    :      ::
H  YLFNWIWRYHFEGFFDL-IAIVAGLVQTVLYCDFFYLYIT--KVLKGKKL-SLPA
   :  ::: ::  :      ::   :: :: :: :::: :  :    :      ::
S  YIPNWIWRYSTEDKKLDKIAFFAGLLQTLLYSDFFYIYYT--KVIRGKGF-KLPK
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FIG. 1. Sequence comparison of *Arabidopsis* (A), human (H) [erd2.1 (26)], and *S. cerevisiae* (S) (24) proteins. Colons indicate amino acid residues identical in all three proteins. Hyphens mark gaps introduced for maximal homology; numbering includes the gaps. Underlined are amino acids apparently involved in ligand specificity. A potential glycosylation site is indicated by a star.

shown in Fig. 1. The *Arabidopsis* protein exhibits 52% identity with the human homologue and is 49% identical with the *S. cerevisiae* protein, while the human and yeast proteins share 51% identity. Thus, it appears that the divergence of the three proteins is almost equal. Amino acids 51–56 (underlined in Fig. 1) have been shown to be important for the determination of ligand specificity by analysis of chimeric Erd2 proteins from two different yeasts, *S. cerevisiae* and *K. lactis* (44). Moreover, *in vivo* and *in vitro* studies with the human Erd2 protein have confirmed that the specific sequence in this region determines the affinity to the KDEL sequence (27, 31, 32). It is interesting that the amino acid residues in this region of the *Arabidopsis* Erd2 protein are identical to those of the human protein [erd2.1 (26)], except for a conservative change of an aspartate to an asparagine at position 56 (in the underlined region in Fig. 1). This observation is consistent with the fact that retention signals in plants and mammalian cells share a common epitope (16, 17) and have similar sequences (15, 45). There is one putative N-linked glycosylation site (star in Fig. 1) in the *Arabidopsis* homologue that is not present in the human or yeast proteins. Biochemical data have indicated that the yeast and mammalian Erd2 proteins are membrane-associated (24, 29, 31). A comparison of hydropathy profiles (Fig. 2) reveals that the yeast, human, and *Arabidopsis* Erd2 proteins contain seven distinct hydrophobic stretches that are believed to be transmembrane domains.

Southern and Northern Blot Analyses. To determine the number of related genes in the *Arabidopsis* genome, Southern blot analysis was performed (Fig. 3). A single band was detected in Southern blots of *Arabidopsis* genomic DNA digested with either *Hin*dIII, *Cla* I, or *Cla* I/*Hin*dIII; two bands were observed after digestion with either *Sac* I/*Hin*dIII or *Sac* I, as expected because *Sac* I cuts once at the amino-terminus of the coding region in the cDNA. A similar banding pattern was seen with both high- and low-stringency hybridization conditions. Thus, there appears to be a single gene for *aERD2* in *Arabidopsis*, differing from the situation in human cells, which have two genes (26, 27). In addition, the difference in length between a fragment of 600 bp formed when the cDNA was digested with *Cla* I/*Hin*dIII and a hybridizing band of ≈2.1 kb in genomic DNA digested with the same enzymes suggested that the coding region is probably interrupted by at least one intron. A 1.0-kb mRNA, similar to the size of the cDNA clone, was detected by

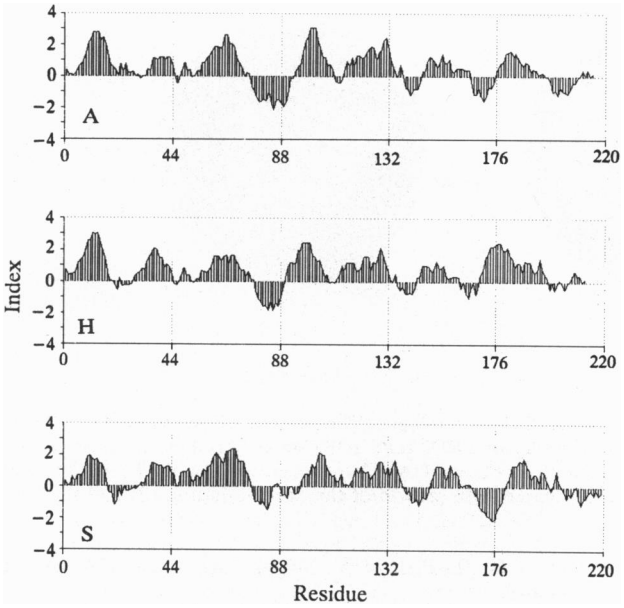

FIG. 2. Hydropathy plots of *Arabidopsis* (A), human (H), and *S. cerevisiae* (S) proteins. The algorithm of Kyte and Doolittle was used (35). Hydrophobic portions are above the horizontal line.

hybridization of total RNA from various tissues with a ^{32}P-labeled probe of the *aERD2* coding region (Fig. 4). Expression of the *aERD2* mRNA was detected in all tissues examined, although the transcript was expressed to a lesser degree in mature leaves than in flower buds, and to a higher degree in stems and roots (Fig. 4). The low level of expression of the protein in leaves is surprising, as the protein is known to be essential in yeast (24), but may indicate that mature tissues do not require active sorting of ER proteins.

***Arabidopsis ERD2* Functionally Complements the Yeast *erd2* Mutant.** To confirm the function of the plant *aERD2* homologue, we cloned the gene in sense and antisense orientation

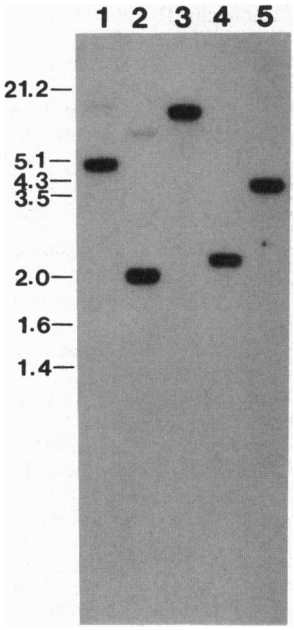

FIG. 3. Southern blot analysis of the *aERD2* gene. *Arabidopsis* genomic DNA from ecotype RLD (3 μg) was digested with *Sac* I (lane 1), *Sac* I/*Hin*dIII (lane 2), *Hin*dIII (lane 3), *Cla* I/*Hin*dIII (lane 4), or *Cla* I (lane 5). The blot was probed with a *Cla* I–*Hin*dIII DNA fragment corresponding to the coding region of *aERD2*. Size markers at left are in kilobases.

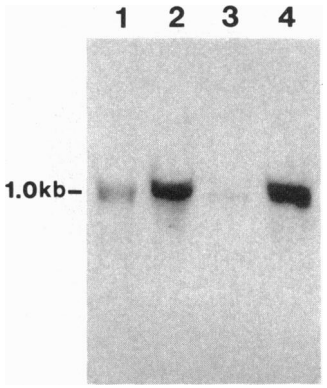

FIG. 4. Northern blot analysis of *aERD2* transcripts. Equal amounts (30 μg) of total RNA from flower buds (lane 1), stems (lane 2), leaves (lane 3), and roots (lane 4) were loaded onto a 1.5% agarose gel containing 6% formaldehyde. The blot was hybridized with ^{32}P-labeled *Cla* I–*Hin*dIII DNA fragment encompassing the coding region of *aERD2*.

in a yeast expression vector containing the *LEU2* gene, and these constructions were transformed into a *S. cerevisiae* strain lacking the chromosomal copy of *ERD2*. Since the *ERD2* gene is essential for cell viability in yeast (24), this strain is maintained by the presence of the *ERD2* gene from *K. lactis* on a *URA3* plasmid (24, 40). Loss of *URA3* is required for growth of yeast on plates containing FOA (42) and in this strain is possible only if an active complementing gene for *ERD2* is provided. As shown in Fig. 5, yeast transformed with the plant *ERD2* gene in the sense orientation were able to grow on FOA plates, as did the yeast transformed with the *S. cerevisiae ERD2* gene. But neither yeast expressing the plant gene in the antisense orientation nor the parental strain were able to grow on FOA plates. To confirm the loss of the *URA3* plasmid, the strains growing on FOA plates were checked for growth on plates lacking uracil. The strains that were transformed with the *LEU2* plasmid were able to grow on plates lacking leucine, but only the strain containing the plant *ERD2* gene in the antisense orientation was able to grow without uracil (Fig. 5). These results have also been confirmed in a sectoring assay (ref. 40; H. R. B. Pelham, personal communication). Thus, unlike the human homologue, the plant gene we have isolated can complement the loss of *ERD2* gene products in yeast.

Is the *Arabidopsis ERD2* Gene the Functional Link Between Yeast and Mammals? Based on the sequence comparison of the proposed ligand-specificity region and the carboxyl-terminal sequences of plant, mammalian, and yeast proteins retained in the ER, it appeared that the plant and mammalian receptors are more closely related. However, the fact that the plant receptor, unlike the mammalian one, was able to complement the yeast *erd2* mutant suggests that the plant gene is more related to yeast. It is therefore pertinent to discuss possible reasons why the human gene might not function in yeast. It is possible that the human homologue is not as efficiently expressed as the plant gene in yeast due to differences in the codon usage or the environment of the initiation codon, both known to affect heterologous gene expression (46, 47). The human protein could be less abundant than the plant protein because the message or protein is less stable. Another possible explanation for this discrepancy is variations in the efficiency of translocation into the ER or correct folding of the human and plant Erd2 proteins in the yeast ER membrane, although many different mammalian and plant genes containing signal sequences have been tested in yeast and are correctly translocated (2, 8, 48–51).

Alternatively, there may be functional differences between the plant and human proteins in either of the two roles of the

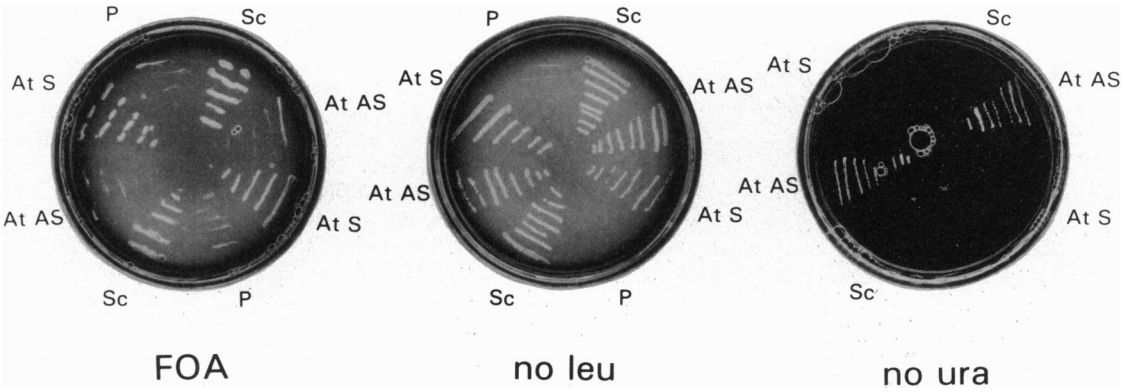

FIG. 5. Complementation of the yeast *erd2* deletion mutant with the *Arabidopsis ERD2* homologue. The parental yeast strain, ΔLE26A (P), and the strain transformed with either the *S. cerevisiae ERD2* gene (Sc) or the *Arabidopsis ERD2* gene in the sense (At S) or antisense (At AS) orientation in the *LEU2*-containing plasmid pRSCErd2 were tested on a plate containing FOA (FOA). Colonies growing on the FOA plate were tested for growth on minimal plates lacking leucine (leu) or uracil (ura) and compared with growth of the parental strain (P) and the yeast transformed with the *Arabidopsis* gene in the antisense orientation (At AS).

Erd2 receptor, binding and retrieval of ER-resident proteins, manifested as slightly different efficiencies of retention of these proteins. In *S. cerevisiae*, there is a strict requirement for a carboxyl-terminal HDEL for ER retention (25). In animals and plants, many different carboxyl-terminal sequences on ER-resident proteins are tolerated, including H/K/RDEL, and a variety of other sequences often with two or three residues in common with this typical sequence (15, 45). However, the human receptor shows marked differences in affinity for these different sequences in *in vitro* binding assays (31), which may affect the ability of this protein to functionally complement the yeast *erd2* mutation. Although the primary region apparently controlling ligand specificity, amino acids 51–56, is nearly identical between the plant and human [human gene erd2.1 (26)] Erd2 proteins, binding studies with the plant homologue should be performed to determine whether the sequence preference is similar, especially as recent evidence indicates that there may be other residues affecting the binding to a KDEL-containing peptide *in vitro* (32). As to the retrograde transport function of the Erd2 proteins, several regions in the human Erd2 protein are apparently required for the recycling of the receptor (32), including Asp^{193}, which is conserved in the *Arabidopsis* Erd2 protein as Asp^{196}. The differences between the *ERD2* homologues from plants and humans in the functional activity in yeast are likely to be reflected in the amino acid sequences of the two proteins. Mutational analysis and fusion constructs between the different *ERD2* genes, followed by complementation tests, binding, and localization studies, will assist in the mapping of the functional domains of the Erd2 proteins.

We thank H. R. B. Pelham for providing the yeast strains and yeast expression plasmid, K. Kerekes for assistance in sequencing, S. Aldrich for help with the yeast work, H. R. B. Pelham and J. Denecke for helpful discussions, and M. Bar-Peled, G. Hicks, and R. Varagona for critical reading of the manuscript. This work was supported by Grant DE-FG02-91ER20021 from the United States Department of Energy to N.V.R.

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