Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4129–4133, May 1995
Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

The ethylene hormone response in *Arabidopsis*: A eukaryotic two-component signaling system

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ABSTRACT **The simple gas ethylene affects numerous physiological processes in the growth and development of higher plants. With the use of molecular genetic approaches, we are beginning to learn how plants perceive ethylene and how this signal is transduced. Components of ethylene signal transduction are defined by ethylene response mutants in *Arabidopsis thaliana*. The genes corresponding to two of these mutants, *etr1* and *ctr1*, have been cloned. The *ETR1* gene encodes a homolog of two-component regulators that are known almost exclusively in prokaryotes. The two-component regulators in prokaryotes are involved in the perception and transduction of a wide range of environmental signals leading to adaptive responses. The *CTR1* gene encodes a homolog of the Raf family of serine/threonine protein kinases. Raf is part of a mitogen-activated protein kinase cascade known to regulate cell growth and development in mammals, worms, and flies. The ethylene response pathway may, therefore, exemplify a conserved protein kinase cascade regulated by a two-component system. The dominance of all known mutant alleles of *ETR1* may be due to either constitutive activation of the ETR1 protein or dominant interference of wild-type activity. The discovery of *Arabidopsis* genes encoding proteins related to ETR1 suggests that the failure to recover recessive *etr1* mutant alleles may be due to the presence of redundant genes.**

The simple gas ethylene (C_2H_4) serves as a plant hormone with profound effects on plant growth and development (reviewed in ref. 1). The best known effect is the induction of ripening in climacteric fruits such as tomatoes. In addition to ripening, there are numerous responses to ethylene throughout the plant during most stages of development. These responses include promotion of seed germination, promotion or inhibition of flowering, abscission of various organs, and senescence. The biosynthesis of ethylene is highly regulated and is under both positive and negative feedback control (reviewed in ref. 2). External stresses such as wounding, pathogen invasion, and flooding can induce ethylene biosynthesis; stress-induced ethylene, in turn, leads to defense responses such as accelerated senescence, abscission of infected organs, or induction of specific defense proteins (1).

The biosynthetic pathway for ethylene and aspects of its regulation have been established in the last decade, and key ethylene biosynthetic genes have been cloned (reviewed in ref. 3). A long-standing question, however, remains: How do plants recognize this simple gaseous molecule and transduce the signal, which leads to complex physiological responses? It is generally believed that ethylene is perceived by a receptor and that transduction of the ethylene signal leads to changes in gene expression. It is established that gene expression patterns change in response to ethylene, and a number of ethylene-induced genes have been cloned (1). Regarding transduction of the signal, it is known that protein phosphorylation (4) and calcium (5) are involved in at least the pathogenesis response to ethylene. In addition, receptors for ethylene have been pursued through studies of ethylene binding in plant extracts (reviewed in ref. 6); however, the observed binding has not been associated with physiological responses.

With the exception of the above, the mechanisms of ethylene perception and signal transduction were essentially unknown until the recent application of molecular genetic approaches to the problem. In *Arabidopsis thaliana*, a genetic dissection of the ethylene response pathway is provided by various ethylene response mutants (Table 1) (13). Mutants have been isolated using a simple screen (13) based on the "triple response" of dark-grown, ethylene-treated seedlings which was first described in pea nearly a century ago (14). In *Arabidopsis*, the triple response consists of inhibition of hypocotyl and root elongation, radial swelling of the hypocotyl, and retention and accentuation of the apical hook (7, 8). The purpose of these dramatic physical changes might be to facilitate soil penetration (15). The altered form conceivably improves the lifting capacity of the shoot while protecting the delicate shoot apex. Ethylene is produced by germinating seedlings in response to physical impedance, which is a condition met during emergence from the soil (15). In the laboratory, tens of thousands of germinating *Arabidopsis* seeds in a single Petri dish can be exposed to exogenous ethylene and then screened for lack of the triple response. A number of mutants that fail to respond to ethylene have been isolated by this method (7, 8, 11, 12). In addition, by screening in the absence of ethylene for presence of the triple response, mutants that either constitutively respond to ethylene (10) or overproduce ethylene (8, 10) have been obtained.

The genes responsible for two of these mutants, *etr1* (9) and *ctr1* (10), were recently cloned. The DNA sequences of these genes hint at the involvement of certain molecular events in ethylene perception and signal transduction. We present here our current understanding of those events and our current hypotheses.

The *ETR1* Gene

There are four known mutant alleles of the *ETR1* locus in *Arabidopsis* (7–9). All mutant alleles confer insensitivity to ethylene and are dominant to the wild-type allele. One of these mutants was examined for a variety of ethylene responses, including seed germination, the triple response, ethylene biosynthesis, and peroxidase activity in leaves and stems, and, in

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Abbreviation: MAP kinase, mitogen-activated protein kinase.

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Table 1. Ethylene-related mutants in *Arabidopsis*

Mutant	Ethylene-response phenotype	Chromosomal position	Comments	Ref(s).
etr1 (ein1)	Insensitive	1, bottom	Dominant, gene cloned	7–9
ein2	Insensitive	5, top	Recessive, pathogen tolerance, gene cloned	8, *
ein3	Insensitive	3, top	Recessive, weaker phenotype, epistatic to *ctr1*, gene cloned	10, †
etr4	Insensitive	3, top	Dominant	‡
ain1	Insensitive	1, middle	Recessive, weaker phenotype	11
eti	Insensitive	Unknown	Five mutants, not characterized genetically	12
ctr1	Constitutive	5, top	Recessive, epistatic to *etr1*, gene cloned	10
eto1	Constitutive	3, bottom	Recessive, ethylene overproduced	8
eto2	Constitutive	5, bottom	Dominant, ethylene overproduced	10
eto3	Constitutive	3, bottom	Dominant, ethylene overproduced	10
hls1	Apical hook absent in triple response	4, bottom	Recessive, gene cloned	8, §

*Gregg Roman and Joe Ecker, personal communication.
†Madge Rothenberg and Joe Ecker, personal communication.
‡Quinhong Chen and Anthony Bleecker, personal communication.
§Anne Lehman and Joe Ecker, personal communication.

all instances, the mutant failed to show response to ethylene (7). The fact that a single mutant lacks a range of ethylene responses in different tissues and developmental stages implies that diverse ethylene responses have at least one regulatory step in common. ETR1, therefore, appears to be a central component of ethylene responses. One possibility is that ETR1 is a receptor for ethylene. This hypothesis is supported by the fact that ethylene binding measured in one of the mutants was only one-fifth of that in the wild type (7).

The *Arabidopsis ETR1* gene was cloned by chromosome walking (9). The deduced ETR1 protein contains 738 amino acids. Each of the *etr1* mutant alleles contains a missense mutation: the mutations affect amino acid residues 31, 62, 65, and 102, respectively. These mutations lie within the three potential membrane-spanning domains of the deduced protein. The first 320 amino-terminal residues have no significant similarity to sequences in current data bases. However, the remaining portion is highly similar to a large family of environmental signal transducers known as two-component regulators (Fig. 1) (reviewed in ref. 16). This family is widespread in prokaryotes; numerous two-component genes have been isolated from a variety of bacterial species. The two-component regulators in prokaryotes control a broad range of adaptive responses to environmental stimuli, including chemotaxis, host recognition and invasion, adaptation to osmolarity, and metabolic changes in response to nitrogen, carbon, and phosphate. Although these regulators are prevalent in prokaryotes, *ETR1* is the only clear two-component homolog currently known among higher eukaryotes (17). In the lower eukaryote *Saccharomyces cerevisiae*, there are three two-component homologs that have recently been discovered (18–20). In addition, less conserved sequence similarities have been noted in plant phytochrome (21) and rat branched-chain α-ketoacid dehydrogenase kinase (22).

In prokaryotes, the protein domains coding for sensors and response regulators are arranged in a variety of configurations (16). For example, the sensors and response regulators can be together in a single protein. The deduced ETR1 protein has one such arrangement in which the protein contains a sensor followed by a receiver, but lacks an output domain.

Emerging Picture of Ethylene Signal Transduction

A Two-Component System. How might ETR1 function in ethylene signal transduction? The similarities between two-component regulators and the deduced ETR1 protein suggest the possibility that ETR1 behaves as a sensor component for ethylene. It is plausible that the amino-terminal domain of ETR1 perceives ethylene and transduces the signal through the phosphotransfer mechanism established for many bacterial sensors and receivers (Fig. 1) (16). The manner in which ethylene is perceived remains unknown. The ETR1 protein may either bind ethylene or sense ethylene indirectly through additional components. It has not been determined whether ETR1 binds a transition metal, but any ethylene binding site is predicted to contain a transition metal such as Zn^{2+} or Cu^{2+}, based on the affinity of alkenes for metals (23, 24). In prokaryotes, only a handful of the sensor proteins have known functions, and in those few cases there are examples of both direct and indirect ligand activation.

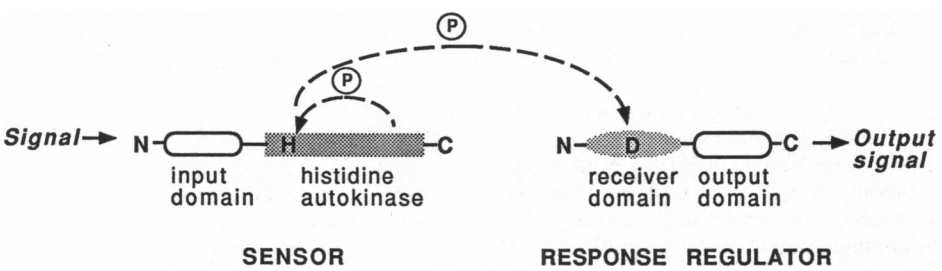

FIG. 1. Basic scheme of the two-component system. The two components are a sensor and a response regulator. The sensor generally consists of a variable amino-terminal or "input" domain (situated in the periplasmic space of the prokaryotic cell) and a conserved carboxyl-terminal histidine protein kinase domain (located in the cytoplasm). When the input domain of the sensor perceives an appropriate signal, autophosphorylation by the histidine protein kinase domain occurs. The associated second component, which is a response regulator, consists of a conserved receiver domain and a variable "output" domain. (Many of the bacterial response regulators have output domains that are transcriptional activators.) The phosphate group on the histidine of the sensor is transferred to a conserved aspartate in the receiver domain of the cognate response regulator. Phosphorylation on this aspartate regulates the activity of the attached output domain leading to adaptive responses.

To complete the analogy with bacterial two-component circuits, the expected substrate for ETR1 should be a response regulator protein consisting of a receiver domain and an output domain. An output domain would be necessary to modulate downstream activities in the ethylene signaling pathway depending on the phosphorylation state of the receiver domain. A clearer picture of the pathway should emerge as the genes of other ethylene response loci (e.g., in Table 1) are isolated.

A Phosphorylation Cascade. Mutants at the *Arabidopsis CTR1* locus display constitutive ethylene response in both seedlings and mature plants (10). These mutants, which are all recessive to the wild-type allele, behave as though they are constantly exposed to ethylene, even when ethylene is not present (10). This suggests that the wild-type CTR1 protein is a negative regulator of ethylene response. CTR1 is believed to act downstream of ETR1 based on genetic crosses showing that mutations in *CTR1* are epistatic to mutations in *ETR1* (10).

The *Arabidopsis CTR1* gene was cloned by T-DNA tagging (10). The deduced protein sequence of CTR1 indicates that it is a protein kinase most similar to the Raf family of serine/threonine protein kinases. Raf protein kinases are part of mitogen-activated protein kinase (MAP kinase) cascades that regulate cell differentiation and growth in mammals, worms, and flies (25). These pathways initiate at the cell surface with tyrosine protein kinases (often transmembrane receptors) that regulate the oncoprotein Ras, which, in turn, binds to and controls the activity of Raf (26).

The sequence similarities between *CTR1* and the Raf family of protein kinases would suggest that the ethylene response pathway might be similar to the ubiquitous Ras pathway found in other eukaryotes. The inclusion of *ETR1* in the picture, however, suggests that the ethylene response pathway regulates a MAP kinase cascade in a different way. Conceivably, there is a direct interaction between ETR1 and CTR1, particularly in light of the observation that the three-dimensional structure of Ras is remarkably similar to that of the two-component receiver domain (27, 28). It is possible, however, that the direct target of ETR1 is a response regulator whose output domain regulates a phosphorylation cascade. In any case, the ethylene response pathway is likely to involve a number of additional components not found in the bacterial two-component system.

The Osmolarity Response Pathway in Yeast. A framework for understanding the ethylene response pathway in plants has recently been provided by a postulated pathway for osmolarity response in yeast (Fig. 2). Other than *ETR1*, the two-component homologs known to date in eukaryotes are the *S. cerevisiae* genes *SLN1* (18), *SSK1* (19), and *SKN7* (20). The deduced SLN1 protein, like ETR1, consists of a novel amino-terminal domain followed by both a putative histidine protein kinase domain and a putative receiver domain. The amino-terminal domain of SLN1, which is flanked by two potential transmembrane domains, is larger than the amino-terminal domain of ETR1. The two domains share no sequence similarity. *SSK1* and *SKN7* do not encode sensor components, but they do encode putative response regulators. Both of the deduced SSK1 and SKN7 proteins are comprised of a novel amino-terminal "output" domain followed by a typical receiver domain. The function of *SKN7* has not been determined (20).

Mutants of *SLN1* have a lethal phenotype that can be suppressed by mutations in other genes. An analysis of extragenic *sln1* suppressors has led to the association of *SLN1* with *SSK1* and an osmolarity response pathway in yeast (19). The *SSK1* gene was first identified as a suppressor of *sln1* (19),

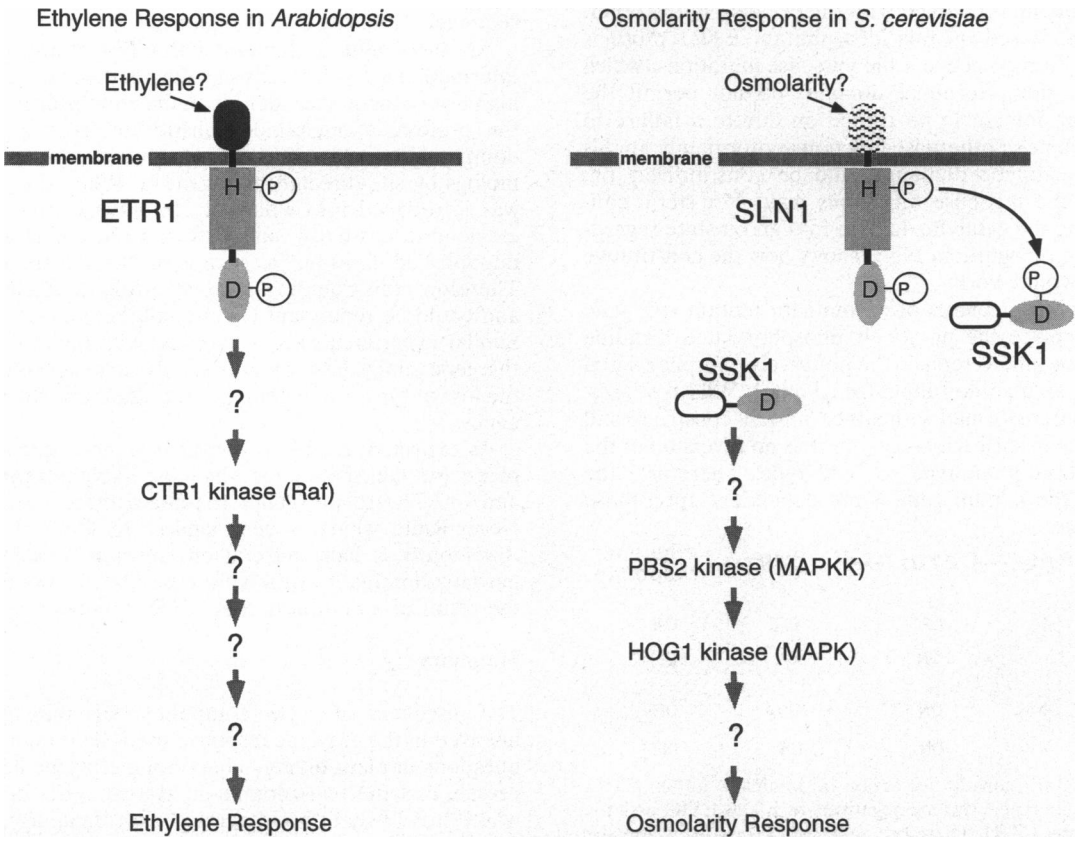

FIG. 2. The two-component system in eukaryotes. In *Arabidopsis*, the two-component regulator ETR1 may control CTR1 activity in response to ethylene (9). CTR1, which is a negative regulator of ethylene response, has sequence similarities with Raf, a MAP kinase kinase kinase (10). In yeast, the two-component regulator SLN1 (possibly a dimer) may react to low osmolarity by autophosphorylation on the histidine, followed by transfer of the phosphate to the aspartate of the response regulator SSK1 (19). The unphosphorylated, active form of SSK1 activates a MAP kinase cascade leading to osmolarity response (19).

which suggests that the SSK1 protein acts downstream of SLN1. SLN1 presumably phosphorylates the SSK1 receiver domain on the conserved aspartyl residue. Analysis of two other *sln1* suppressor genes, *PBS2* and *HOG1*, led to the discovery that the SLN1/SSK1 two-component pair acts upstream of a MAP kinase cascade (19). *PBS2* (29) and *HOG1* (30) encode a MAP kinase kinase and a MAP kinase, respectively. In addition, *PBS2* and *HOG1* are known to regulate glycerol synthesis in response to high osmolarity (30). This regulation was shown to be dependent on *SSK1* (19).

The picture of the yeast osmolarity response pathway is quite similar to that emerging for the ethylene response pathway in plants. Both involve similar two-component homologs and contain components of MAP kinase cascades (Fig. 2). Interestingly, two mammalian homologs of HOG1 were recently cloned, and they are both involved in osmolarity responses in mammalian cells (31, 32).

Genetic Basis of ETR1 Function

Basis for Dominance. A complete understanding of a gene's function requires knowledge of the phenotype when the gene product is absent. For the *ETR1* gene, the loss of function phenotype remains undetermined because all known mutant alleles are dominant and are missense mutations. It is possible, nonetheless, to gain some understanding of ETR1 function by examining the basis for the dominance of the mutant alleles. There are two hypotheses for why *etr1* mutant alleles are dominant. The mutant ETR1 proteins may be constitutively active or may be participating in dominant interference.

In the bacterial two-component system, there is evidence that the amino-terminal signal-sensing domain acts oppositely with the histidine protein kinase domain (16). That is, the on state of one domain is coupled with the off state in the other, and vice versa. Based on this idea, mutant ETR1 proteins could be constitutively active if the missense mutations (which lie within the amino-terminal domain) do not permit the amino-terminal domain to be turned on due to a failure to recognize ethylene or otherwise function appropriately. In this situation, the catalytic domain would be constitutively on. Alternatively, the missense mutations may alter steric constraints, locking the catalytic domain into an on state regardless of ethylene recognition. Fig. 3 shows how the constitutive action model would work.

Site-directed mutagenesis of a dominant mutant *etr1* gene was used to replace the putatively phosphorylated histidine with a glutamine and to replace the putatively phosphorylated aspartate with an alanine (unpublished data). Wild-type *Arabidopsis* plants transformed with either of these constructs still display ethylene insensitivity—i.e., there is no reversion of the dominant mutant phenotype to wild type. Therefore, the dominance of the mutant gene is not dependent upon those

	C_2H_4 —|	*ETR1* →	*CTR1* —|	ethylene response
wild type	+	OFF	OFF	ON
	-	ON	ON	OFF
etr1 dominant mutant	+	ON	ON	OFF
	-	ON	ON	OFF

FIG. 3. Model for constitutive action of dominant mutant ETR1 protein. In the wild type, ethylene negatively regulates ETR1 activity, and ETR1 activates CTR1. Therefore, in the presence of ethylene, the wild-type ETR1 protein is off, and thus, the negative regulator CTR1 is off, permitting ethylene responses. In the *etr1* mutant, the defective protein either fails to recognize ethylene or is sterically locked into an active state. Therefore, in the presence of ethylene, the mutant protein activates CTR1, which in turn represses ethylene responses.

particular histidine and aspartate residues. These results argue somewhat against the constitutive activation model.

The other explanation for the dominance of the *etr1* mutant alleles is dominant interference (33). In other words, the mutant proteins are not necessarily active, but they interfere somehow with the wild-type ethylene response. For instance, if ETR1 acts as a homodimer, then wild-type copies form an active dimer, and the presence of mutant copies may interfere with either multimer formation or activity.

Redundant Proteins? A further understanding of the role of *ETR1* in the ethylene response can be obtained by determining why recessive *etr1* mutant alleles have not been obtained. Presumably, missense or nonsense mutations in *ETR1*, which would eliminate or reduce ETR1 activity, are able to occur. In the yeast *SLN1* gene, for instance, a missense mutation in the putative ATP-binding motif results in a loss of SLN1 activity. Yet, so far, such mutations at the *ETR1* locus have not been found in screens for ethylene response mutants. There are several possible explanations for this result. Loss of ETR1 function may be lethal or may require a screen for another phenotype. Alternatively, there may be no detectable phenotype. For example, a wild-type phenotype may result if there are proteins with functions that are redundant with ETR1 function.

Low stringency hybridization of the *ETR1* gene to *Arabidopsis* genome blots indicates there may be a family of *ETR1*-related genes in *Arabidopsis* (unpublished data). To date, two *ETR1* homologs have been isolated and characterized in *Arabidopsis* (Jian Hua, C.C., Qi Sun, and E.M.M., unpublished data; Hajime Sakai and E.M.M., unpublished data). One of these encodes a putative histidine protein kinase domain but lacks a receiver domain. The predicted amino-terminal domains of both genes are highly conserved with *ETR1* even though the methods used to isolate them were not based on sequence similarity in this region.

Are these genes redundant with *ETR1* or are they common intermediates for different signaling pathways? Because of the high level of sequence identity in the amino-terminal region of the protein, a nucleotide substitution corresponding to a dominant *etr1* mutation was introduced into one of the homologs by site-directed mutagenesis. When the altered gene was introduced into wild-type *Arabidopsis* plants, dominant ethylene insensitivity was conferred (Jian Hua and E.M.M., unpublished data) just as seen with the *etr1* mutant gene (9). Therefore, this homolog can play a role in ethylene response and could be redundant (or partially redundant) with *ETR1*. Similar experiments are in progress with the third member of this gene family. Experiments are also in progress to determine the loss of function phenotypes for *ETR1* and the homologous genes.

As expected, *ETR1* cross-hybridizes to sequences in other plants (unpublished data) which are likely to represent *ETR1* and/or *ETR1*-related genes. In tomato, there is a mutant called Never-Ripe, which is very similar to the *etr1* mutants in *Arabidopsis*; it lacks all ethylene responses examined and is partially dominant to the wild type (34). Never-Ripe may be the result of a mutation in an *ETR1* homolog in tomato.

Summary

The discovery of a two-component signaling gene that is involved in the ethylene response sheds light on long-standing questions in plant biology concerning ethylene action. It also reveals that the two-component system exists in eukaryotes, where it is likely to be integrated with signaling circuitry not found in prokaryotes. Plants contain multiple two-component genes, as shown by the isolation of two *ETR1* homologs. The high level of sequence identity between the amino-terminal domains of ETR1 and both homologs indicates that the three proteins may have similar functions. Each of the *etr1* mutant

alleles carries a missense mutation in this domain, and when one of these mutations was introduced into one of the homologs, the homolog conferred dominant ethylene insensitivity to wild-type *Arabidopsis* plants.

The two-component system may prove to be as widely utilized in plants as in prokaryotes. Alternatively, ethylene signaling in plants may be a rare example. It will be interesting to see whether the two-component gene family extends into animals or whether the two-component system is limited to bacteria, fungi, and plants.

We thank Anthony Bleecker and Joe Ecker for permission to cite unpublished data from their laboratories. We thank J. Goodrich, J. Hua, S. Jacobsen, B. Krizek, J. Levin, Z. Liu, M. Running, and H. Sakai for comments on the manuscript. Our work on ethylene response is supported by U.S. Department of Energy Grant FG03-88ER13873.

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