



(86) Date de dépôt PCT/PCT Filing Date: 1999/11/24

(87) Date publication PCT/PCT Publication Date: 2000/06/08

(85) Entrée phase nationale/National Entry: 2001/05/24

(86) N° demande PCT/PCT Application No.: US 99/28104

(87) N° publication PCT/PCT Publication No.: WO 00/32761

(30) Priorité/Priority: 1998/11/25 (60/109,973) US

(51) Cl.Int.⁷/Int.Cl.⁷ C12N 15/01, A01H 4/00, A01H 5/00,
C12P 21/02, C12P 21/08, C12N 5/04, C12N 15/82,
C12N 15/62

(71) Demandeur/Applicant:
THE TRUSTEES OF THE UNIVERSITY OF
PENNSYLVANIA, US

(72) Inventeurs/Inventors:
SOLANO, ROBERTO J., ES;
ECKER, JOSEPH R., US

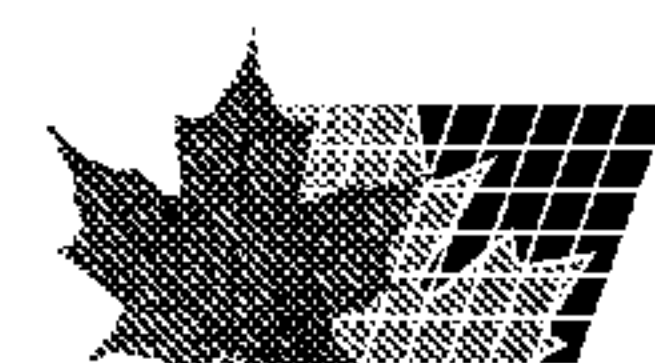
(74) Agent: BERESKIN & PARR

(54) Titre : FACTEUR 1 DE REPONSE ETHYLENIQUE (ERF1) DANS DES VEGETAUX

(54) Title: ETHYLENE-RESPONSE-FACTOR1 (ERF1) IN PLANTS

(57) **Abrégé/Abstract:**

The invention provides an isolated nucleic acid encoding Ethylene Response Factor 1 (ERF1), an early responsive gene, encoding a GCC-box binding protein in the ethylene gas signaling pathway in plants. Also provided and characterized is the peptide ERF1, as well as methods of using ERF1 or its expression product to modulate the response in plants or plant cells to ethylene. Moreover, because ERF1 acts downstream of EIN3 and all previously identified members of the signaling pathway, and because constitutive expression of ERF1 results in the modulation of a variety of ethylene response genes and phenotypes, the invention provides novel and useful methods for regulating the ethylene signaling pathway.



PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/01, 15/62, 15/82, 5/04, A01H 4/00, 5/00, C12P 21/02, 21/08	A1	(11) International Publication Number: WO 00/32761 (43) International Publication Date: 8 June 2000 (08.06.00)
(21) International Application Number: PCT/US99/28104 (22) International Filing Date: 24 November 1999 (24.11.99) (30) Priority Data: 60/109,973 25 November 1998 (25.11.98) US (71) Applicant (for all designated States except US): THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ECKER, Joseph, R. [US/US]; 6 Hastings Drive, Mullica Hill, NJ 08062 (US). SOLANO, Roberto, J. [ES/ES]; Centro Nacional de Biotecnología-CSIC, Campus Cantoblanco. Ctra., Colmenar KM 15.5, E-28049 Madrid (ES). (74) Agent: McCONATHY, Evelyn, H.; Dilworth Paxson LLP, 3200 Mellon Bank Center, 1735 Market Street, Philadelphia, PA 19103-7595 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: ETHYLENE-RESPONSE-FACTOR1 (ERF1) IN PLANTS (57) Abstract The invention provides an isolated nucleic acid encoding <i>Ethylene Response Factor 1 (ERF1)</i> , an early responsive gene, encoding a GCC-box binding protein in the ethylene gas signaling pathway in plants. Also provided and characterized is the peptide ERF1, as well as methods of using <i>ERF1</i> or its expression product to modulate the response in plants or plant cells to ethylene. Moreover, because <i>ERF1</i> acts downstream of <i>EIN3</i> and all previously identified members of the signaling pathway, and because constitutive expression of <i>ERF1</i> results in the modulation of a variety of ethylene response genes and phenotypes, the invention provides novel and useful methods for regulating the ethylene signaling pathway.		

ETHYLENE-RESPONSE-FACTOR1 (ERF1) IN PLANTS

REFERENCE TO RELATED APPLICATIONS

- 5 This application claims priority to US Provisional Application 60/109,973, filed November 25, 1998.

FIELD OF THE INVENTION

- 10 The invention relates to the Ethylene-Response-Factor1 (ERF1) and its function in the ethylene gas signaling pathway in plants. The existence of a hierarchy of enzymes in the biosynthetic and signaling pathways provides a means to finely regulate the complex plant response to ethylene as a growth or maturation regulator and/or stress or injury signal.

15 GOVERNMENT INTERESTS

 This invention was supported in part by the National Science Foundation grant number MCB-95-07166. The Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

- 20 The plant hormone ethylene (C₂H₄) regulates a variety of stress responses and developmental adaptations in plants. This gaseous molecule is well known for its participation in physiological processes as diverse as fruit ripening, senescence, abscission, germination, cell elongation, sex determination, pathogen defense response, wounding, nodulation and determination of cell fate (Abeles *et al.* (1992) in Ethylene in
-25 Plant Biology, Academic Press, Inc., New York; Tanimoto *et al.*, *Plant J.* 8:943-948 (1995); Penninckx *et al.*, *Plant Cell* 8:2309-2323 (1996); O'Donnell *et al.*, *Science* 274:1914-1917 (1996); Penmetsa *et al.*, *Science* 275:527-530 (1997)). Understanding the molecular events that lead to this diversity of plant responses is essential to elucidate how ethylene modulates such functions.

- 30 A number of biological stresses are known to induce ethylene production in plants, including wounding, abscission, bacterial, viral or fungal infection, and treatment

WO 00/32761

PCT/US99/28104

with elicitors, such as glycopeptide elicitor preparations from fungal pathogen cells. In the case of abscission, a particular layer of cells in a zone located between the base of the leaf stalk and the stem (the abscission zone) responds to a complex combination of ethylene and other endogenous plant growth regulators by a process that is, to date, not fully understood. However, the effect of abscission is the controlled loss of part of the plant, typically localized; the result of which is visible as a dead leaf or flower, or as softening or "ripening" of fruit, ultimately leaving the plant wounded at the point of separation.

The synthetic pathway of ethylene has been well characterized (Kende, *Plant Physiol.*, 91:1-4 (1989)). The conversion of ACC to ethylene is catalyzed by ethylene forming enzyme (Spanu *et al.*, *EMBO J* 10:2007 (1991)). In a closed circular ethylene synthetic pathway, S-adenosyl-1 methionine (SAM) is produced from methionine. Then, in a rate-limiting step, SAM is converted to 1 aminocyclopropane-1-carboxylic acid (ACC) by an ACC synthase. In a final step, ethylene is produced from ACC by an ACC oxidase. The pathway, therefore, utilizes multiple ACC synthases and ACC oxidases, creating a cascade or hierarchy of enzymes in the biosynthetic pathway and providing a means for regulating the complex plant response.

Scientists are just beginning to understand the ethylene signal transduction pathway at the molecular level. To address the ethylene signaling mechanisms, a molecular/genetic approach has been applied using the ethylene-evoked triple response phenotype of *Arabidopsis thaliana* seedlings. The morphological changes evoked by continuous exposure of *Arabidopsis* seedlings to ethylene are known as the "triple response." Recognition of the triple-response to the hormone has allowed the identification of a number of components of the ethylene response pathway.

Based upon the triple response, a dozen *Arabidopsis* mutants have been isolated into two classes (Ecker, *Science* 268:667-675 (1995); Johnson & Ecker, *Annu. Rev. Genetics* 32:227-254 (1998); US Pat. Nos. 5,367,065; 5,444,166; 5,602,322; 5,650,553; and 5,955,652, each of which is herein incorporated by reference). One class of mutants, the *ein* (ethylene insensitive) mutants, show reduced or complete insensitivity to

WO 00/32761

PCT/US99/28104

exogenous ethylene. The group of insensitive includes *etr1*, *etr2*, *ein2*, *ein3*, *ein4*, *ein5/ain1*, *ein6*, and *ein7* mutants (Ecker, 1995; McGrath and Ecker, *Plant Physiol. Biochem.* 36:103-113 (1998); Sakai *et al.*, *Proc. Natl. Acad. Sci. USA* 95:5812-5817 (1998). Based on epistasis analysis, a genetic framework for the action of these genes

5 has been established (Roman *et al.*, *Genetics* 139, 1393-1409 (1995); Sakai *et al.*, 1998). *ETR1*, *ETR2* and *EIN4* genes act upstream of *CTR1*, whereas *EIN2*, *EIN3*, *EIN5/AIN1*, *EIN6* and *EIN7* genes act downstream of *CTR1*.

The other class of mutants (the constitutive hormone response mutants) display constitutive ethylene response phenotypes in the absence of exogenously applied

10 hormones. Using antagonists of ethylene biosynthesis and activity, this class was further divided into those mutants that display a "constitutive" triple response phenotype as a result of either ethylene overproduction (*eto1*, *eto2* and *eto3*) (Guzman and Ecker, 1990; Kieber *et al.*, *Cell* 72:427-441 (1993)), or constitutive activation of the pathway (*ctr1*; constitutive triple response) (Kieber *et al.*, 1993). The latter mutants display "ethylene"

15 phenotypes even in the presence of inhibitors of ethylene biosynthesis or receptor binding

Based upon a loss-of-function mutation conferring an ethylene constitutive response phenotype, *CTR1* has been identified as a negative regulator of the ethylene response pathway. The *CTR1* gene encodes a protein with similarity to the Raf-family of protein kinases, implicating a MAP-kinase cascade in the ethylene response pathway

20 (Kieber *et al.*, 1993)). Coupling of a "bacterial"-type receptor and "Raf"-like protein kinases in the osmo-sensing pathway in yeast is provided by phospho-relay proteins (Posas *et al.*, *Cell* 86: 865-875 (1996)). While several proteins with both structural and functional similarity to response regulators have been identified in *Arabidopsis* (Imamura

25 *et al.*, *Proc. Natl. Acad. Sci. USA* 95:2691-2696 (1998)), the ethylene receptors *ETR1* and *ERS1* (Chang, *Trends Biochem. Sci.* 21:129-133 (1996); Schaller *et al.*, *Science* 270:1809-1811 (1995)); can physically interact with *CTR1* (Clark *et al.*, *Proc. Natl. Acad. Sci. USA* 95:5401-5406 (1998)); thus by-passing an absolute requirement for such intermediates.

WO 00/32761

PCT/US99/28104

Less is understood about the downstream components of the ethylene signaling pathway. Cloning and characterization of the *EIN3* gene revealed that it encodes a nuclear-localized protein (Chao *et al.*, *Cell* 89:1133-1144 (1997); Solano *et al.*, *Genes Dev.* 12:3703-3714 (1998)). While sequence analysis failed to uncover homology to previously described proteins, EIN3 shares amino acid sequence similarity, conserved structural features and genetic function with three EIN3-LIKE (EIL) proteins. Genetic studies revealed that *EIL1* and *EIL2* were able to functionally complement the *ein3* mutation, suggesting their participation in the ethylene signaling pathway. High level expression of *EIN3* or *EIL1* in transgenic wild-type or *ein2* mutant plants conferred constitutive ethylene response phenotypes in all stages of development, indicating their sufficiency for activation of the pathway in the absence of ethylene.

The most extensively studied of the various classes of ethylene-responsive genes, are the secondary response genes, whose expression is activated by ethylene in response to attack by pathogens. These include basic-chitinases, β -1,3-glucanases, defensins and other pathogenesis-related (PR) proteins (Boller *et al.*, *Planta* 157:22-31 (1983); Felix *et al.*, *Planta* 167:206-211(1986); Broglie *et al.*, *Plant Cell* 1:599-607 (1989); Ohme-Takagi *et al.*, *Plant Mol. Biol.* 15:941-946 (1990); Samac *et al.*, *Plant Physiol.* 93:907-914 (1990); Eyal *et al.*, *Plant J.* 4:225-234 (1993); Penninckx *et al.*, 1996). Analysis of the promoters of several of these genes revealed a common *cis*-acting ethylene response element called the GCC box. This element was shown to be necessary and sufficient for ethylene regulation in a variety of plant species (Eyal *et al.*, 1993; Meller *et al.*, *Plant Mol. Biol.* 23:453-463 (1993); Hart *et al.*, *Plant Mol. Biol.* 21, 121-131 (1993); Shinshi *et al.*, *Plant Mol. Biol.* 27:923-932 (1995); Sessa *et al.*, *Plant Mol. Biol.* 27:923-932 (1995); Ohme-Takagi *et al.*, *Plant Cell* 7:173-182 (1995); Sato *et al.*, *Plant & Cell Physiology* 37:249-255 (1996)).

Efforts to isolate *trans*-acting factors in tobacco that bind the GCC box identified a family of proteins termed Ethylene-Responsive-Element-Binding-Proteins (EREBPs) (Ohme-Takagi *et al.*, 1995). These novel DNA-binding proteins interact *in vitro* with the GCC box through a domain homologous to that previously observed in the floral

WO 00/32761

PCT/US99/28104

homeotic protein APETALA2 (Ecker, 1995; Weigel, *Plant Cell* 7:388-389 (1995)). In *Arabidopsis*, over thirty genes belonging to this family have been identified by several groups (Wilson *et al.*, *Plant Cell* 8:659-671 (1996); Buttner *et al.*, *Proc. Natl. Acad. Sci. USA* 94:5961-5966 (1997); Okamuro *et al.*, *Proc. Natl. Acad. Sci. USA* 94:7076-7081 (1997)) and as result of the *Arabidopsis* Genome Initiative (Bevan *et al.*, *Plant Cell* 9:476-478 (1997), Ecker, *Nature* 391:438-439 (1998)).

The expression of several members of the EREBP family has been reported to be regulated by ethephon, an ethylene releasing compound (Buttner *et al.*, 1997; Ohme-Takagi *et al.*, 1995), suggesting that they may have an intermediary role in the cascade of activators and receptors in the ethylene signaling pathway. However, evidence for the direct involvement of an EREBP in the ethylene signaling pathway in *Arabidopsis* remained lacking until the inventors' discovery of the present invention. Thus, this discovery significantly advances the science and understanding of the ethylene biosynthesis and signaling, and offers means of controlling, modulating or regulating the ethylene response during plant maturation and development, flowering, ripening of fruit, response to stress, injury, pathogens and the like.

By thus improving understanding of the ethylene signal pathway, the present invention enhances the development of methods for improving the tolerance of plants to stress, injury or pathogens, as well as for developing easier and more efficient methods for identifying stress- or injury- or pathogen-tolerant plants. Moreover, by providing insight into plant hormones and mechanisms for their control, and by modulating and regulating their functions, it is possible to significantly improve the quality, quantity and longevity of plant food products, such as fruits and vegetables, flowers and flowering ornamentals, and other non-food plant products, such as commercially valuable crops, e.g., cotton or flax, or ornamental green plants, thereby providing more and better products for market in both developed and underdeveloped countries.

SUMMARY OF THE INVENTION

The present invention relates to the cloning and characterization of *Ethylene Response Factor 1* (*ERF1*), an early responsive gene, encoding a GCC-box binding protein. *EIN3* expression is both necessary and sufficient for *ERF1* transcription. In addition, like *EIN3* over-expression in transgenic plants, constitutive expression of *ERF1* results in the modulation of a variety of ethylene response genes and phenotypes. Moreover, *EIN3* and *EILs* are demonstrated to be novel, sequence-specific DNA-binding proteins that bind a primary response element in the promoter of *ERF1*. Consistent with the findings in the biochemical studies, genetic analysis revealed that *ERF1* acts downstream of *EIN3* and all previously identified members of the ethylene gas signaling pathway.

The present invention provides an isolated nucleic acid encoding a plant ethylene response factor, which is activated by *EIN3* or an *EIN3*-like (*EIL*) peptide in the plant ethylene signaling pathway, wherein the activated factor binds to a target GCC-box in a secondary ethylene response gene. It also provides an isolated nucleic acid comprising *ERF1*, as well mutants, derivatives, homologues or fragments of *ERF1*, encoding an expression product having *ERF1*-activity. It further provides the nucleic acid comprising SEQ ID NO:1.

In addition, the invention provides a purified polypeptide encoded by the above-identified nucleic acids. It also provides a purified polypeptide comprising *ERF1*, as well as homologues, analogs, derivatives or fragments thereof, having GCC-box binding activity in a target secondary response gene. It further provides the polypeptide comprising SEQ ID NO:2, as well as the polypeptide, wherein *ERF1* GCC-box binding activity is *EIN3*- or *EIL*-dependent.

The invention also provides a recombinant cell comprising the above-identified isolated nucleic acids.

It further provides a vector comprising the above-identified isolated nucleic acids.

The invention further provides an antibody specific for a plant *ERF1* polypeptide, and homologues, analogs, derivatives or fragments thereof, having GCC-box binding activity in a target secondary response gene. It also provides an isolated nucleic acid

WO 00/32761

PCT/US99/28104

sequence comprising a sequence complementary to all or part of the *ERF1* nucleic acid sequence, and to mutants, derivatives, homologues or fragments thereof encoding a GCC-box binding expression product in a target secondary response gene. Thus, the invention also provides the nucleic acid having antisense activity at a level sufficient to
5 regulate, control, or modulate the secondary ethylene response activity of a plant, plant cell, organ, flower or tissue comprising same.

The present invention provides a plant, plant cell, organ, flower, tissue, seed, or progeny comprising any of the above identified nucleic acids, including *ERF1*, wherein the plant cells, organs, flowers, tissues, seeds or progeny include those of a transgenic
10 plant. Moreover, the invention is intended to include transgenic plants, plant cells, organs, flowers, tissues, seeds or progeny comprising the aforementioned recombinant nucleic acids or polypeptides.

In addition, the invention provides an isolated nucleic acid, further comprising a plant *ERF1* promoter sequence, or a fragment thereof having *ERF1* promoter activity; as
15 well as a transgenic plant, the cells, organs, flowers, tissues, seed, or progeny of which contain a transgene comprising the *ERF1* promoter sequence.

The invention further provides an isolated *ERF1* nucleic acid, which further comprises a reporter gene operably fused thereto, or a fragment thereof having reporter activity.

20 The invention also provides a method for manipulating in a plant the *ERF1* nucleic acid to permit the regulation, control or modulation of the ethylene response in said plant. It further provides such a method, whereby the regulation, control or modulation results in the initiation or enhancement of germination, cell elongation, sex determination, flower or leaf senescence, flower maturation, fruit ripening, insect,
25 herbicide or pathogen resistance, abscission, or response to stress, injury or pathogens in the plant. In certain embodiments of the invention, the disclosed regulation, control or modulation method provides for the inhibition or prevention of germination, cell elongation, sex determination, flower or leaf senescence, flower maturation, fruit ripening, insect, herbicide or pathogen resistance, abscission, or response to stress, injury
30 or pathogens in the plant. In certain other embodiments of the invention, the disclosed regulation, control or modulation method provides for the activation or enhancement of

WO 00/32761

PCT/US99/28104

germination, cell elongation, sex determination, flower or leaf senescence, flower maturation, fruit ripening, insect, herbicide or pathogen resistance, abscission, or response to stress, injury or pathogens in the plant.

5 The invention also provides a method of identifying a compound capable of affecting the ethylene response in the ethylene signaling system in a plant comprising: providing a cell comprising an isolated nucleic acid encoding a plant ERF1 sequence, having a reporter sequence operably linked thereto; adding to the cell a compound being tested; and measuring the level of reporter gene activity in the cell, wherein a higher or lower level of reporter gene activity in the cell compared with the level of reporter gene
10 activity in a second cell to which the compound being tested was not added is an indicator that the compound being tested is capable of affecting the expression of a plant *ERF1* gene.

In addition, the invention provides a method for generating a modified plant with enhanced ethylene response activity as compared to that of comparable wild type plant
15 comprising introducing into the cells of the modified plant an isolated nucleic acid encoding ERF1, wherein said *ERF1* nucleic acid binds to the GCC-box, thereby activating target secondary ethylene response genes of the modified plant. In certain embodiments of the invention are provided a method for generating a modified plant with enhanced ethylene response activity as compared to that of comparable wild type
20 plant comprising introducing into EIN3- or EIL-defective or deficient cells of the modified plant an isolated nucleic acid encoding EIN3 or EIL, wherein said *EIN3* or *EIL* nucleic acid activates the ERF1 gene, thereby permitting activation of target secondary ethylene response genes of the modified plant.

The invention further provides a method for generating a plant with diminished or
25 inhibited ethylene response activity as compared to that of a comparable wild type plant comprising binding or inhibiting the ERF1 molecules within the cells of the modified plant by introducing into said cells an isolated nucleic acid encoding a complementary nucleic acid to all or a portion of *erf1*, wherein said *erf1* nucleic acid would otherwise bind to the GCC-box, thereby activating target secondary ethylene response genes of the
30 modified plant. In certain embodiments of the invention are provided a method for generating a plant with diminished or inhibited ethylene response activity as compared to

WO 00/32761

PCT/US99/28104

that of a comparable wild type plant comprising binding or inhibiting the ERF1 molecules within the cells of a modified plant by introducing into said cells an antibody to all or a portion of ERF1, wherein said ERF1 polypeptide would otherwise bind to the GCC-box, thereby activating target secondary ethylene response genes of the modified
5 plant.

In certain additional embodiments of the invention are provided a method for generating a plant with diminished or inhibited ethylene response activity as compared to that of a comparable wild type plant comprising binding or inhibiting the EIN3 or EIL molecules within the cells of a modified plant by introducing into said cells an antibody
10 to all or a portion of EIN3 or EIL, wherein said EIN3 or EIL polypeptide would otherwise activate the expression of ERF1, thereby permitting activation of target secondary ethylene response genes of the modified plant.

The invention also provides a method for manipulating the expression of ERF1 in a plant cell comprising: operably fusing the nucleic acid *ERF1* or an operable portion thereof to a plant promoter sequence in the plant cell to form a chimeric DNA, and
15 generating a transgenic plant, the cells of which comprise said chimeric DNA, whereupon by controlling activation of the plant promoter, one can manipulate expression of ERF1

The invention will be more fully understood from the following detailed
20 description of preferred embodiments, drawings and examples, all of which are intended to be for illustrative purposes only, and not intended in any way to limit the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows nuclear events in the ethylene gas signaling pathway. Shown is a
25 model of the transcriptional regulatory cascade that mediates ethylene responses.

Figure 2 sets forth the results of cloning and ethylene inducibility of *ERF1*. Figure 2A depicts the Northern blot analysis of *ERF1* mRNA expression induced in the presence of ethylene gas and its comparison with the induced expression of *PDF1.2*. Figure 2B depicts the Northern blot analysis of the expression of *ERF1* mRNA in *EIN3*-
30 overexpressing plants. Figure 2C depicts the Northern blot analysis of the cycloheximide

WO 00/32761

PCT/US99/28104

induction of *ERF1* expression. Numbers above the lanes indicate μ M concentrations of cycloheximide (cx).

Figure 3 sets forth the data showing that EIN3 is a sequence specific DNA-binding protein. Figure 3A shows the electrophoretic mobility shift assay (EMSA) of EIN3 protein, translated *in vitro*, binding to the -1238 to -950 fragment of the *ERF1* promoter. A control protein (control) or mock translated reticulocyte lysate (RL) was used in the indicated lanes. Figure 3B shows the EMSA of EIN3-3 mutant protein, EIN3 and several deletion derivatives bound to fragments -1238 to -1204 (1) and -1213 to -1178 (2) of the *ERF1* promoter. Figure 3C shows the competition of the interaction of EIN3 with its target site, the EIN3 Binding Site (EBS) by addition of anti-EIN3 antibodies (Ab-EIN3). PI stands for pre-immune serum.

Figure 4 sets forth data to characterize the EBS. Figure 4A depicts scan mutagenesis of the EBS. Wild-type EBS is shown with the palindromic repeats indicated by arrows. Base changes in the mutants tested are indicated. Dots indicate similar bases as in the wild-type EBS (Wt EBS). Figure 4B depicts the sequence alignment of the EBS and a fragment of the promoters of *E4* and *GST1* genes (including the ERE).

Figure 5 sets forth data to characterize the EBS. Figure 5A shows the competition of the EIN3-EBS complex formation by addition of an excess of unlabelled EBS or two mutant versions, EBSm1 and EBSm2. No competitor was added in the lanes marked as 0. Black triangles represent increasing amounts of competitor (20, 60 and 200 nanograms). Figure 5B shows a summary of EIN3 structural features and mutants used in EMSA experiments.

Figure 6 shows EIN3 homo-dimerization. Figure 6A depicts EMSA of full size EIN3 and deletion derivatives binding to the EBS. Figure 6B shows EIN3-EIN3 interactions assayed by the yeast "two-hybrid" system. Yeast cells transformed with the indicated constructs as shown in the top-left plate, were grown on synthetic complete (SC) medium containing histidine (+HIS) or in SC medium without histidine (-HIS) and with 50 mM 3-aminotriazole (3-AT, Sigma) to repress basal activity of the *his3* reporter gene. β -gal activity of the colonies grown in -HIS (lacZ) was determined by the filter-lift assay. SNF4/SNF1 were used as a positive control. Colonies from two independent transformation experiments are shown (BD-EIN3a and BD-EIN3b). Figure 6C depicts

WO 00/32761

PCT/US99/28104

binding of EIL proteins to the EIN3 target site in the *ERF1* promoter. EMSA was performed using *in vitro* translated EIN3, EIL1, EIL2 and EIL3 proteins and the wild-type EBS (W) or mutant (M) version, wherein the mutant exhibits a mutation at position 17 from G to C, as shown in Figure 4.

5 Figure 7 shows data in support of ERF1 as a GCC box DNA binding protein. Electrophoretic mobility shift assays were performed using *in vitro* translated ERF1 protein and promoter fragments from the *Arabidopsis* basic-chitinase (b-CHI) and bean chitinase5B (CH5B) genes. DNA fragments containing the GCC box or mutated versions (b-CHIm and CH5Bm) of these same elements were incubated with control,
10 mock-translated rabbit reticulocyte lysate or those containing EIN3 protein.

Figure 8 depicts constitutive activation of ethylene response phenotypes in *35S::ERF1*-expressing seedlings. Transgenic seedlings overexpressing *ERF1* in wild type (Col-0) and *ein3* mutant backgrounds were grown in continuous flow-through chambers with hydrocarbon-free air in agar with or without 10 μ M ACC. Untransformed
15 wild-type and *ein3* mutant plants are also shown for comparison.

Figure 9 shows that ERF1 acts downstream of EIN2 and EIN3 in the ethylene signaling pathway. Transgenic plants overexpressing *ERF1* in wild type (Col-0), *ein2*, *ein3-1* and *ein3-3* mutant backgrounds were grown in continuous flow-through chambers with hydrocarbon-free air for 5 weeks. Untransformed wild-type, *ctr1-1* and *EIN3*-
20 overexpressing plants are shown for comparison.

Figure 10 shows transcriptional activation of ethylene-responsive genes by ERF1. Figure 10A depicts Northern blot analysis (total RNA) of the expression of ethylene-induced genes in transgenic lines overexpressing *ERF1* in Wt (Col-0) and ethylene insensitive mutant backgrounds. Five independent transgenic lines in Col-0 and 2
25 independent lines in each of the mutants are shown. The same blot was probed with ethylene-induced genes *PDF1.2* and basic-chitinase, *ERF1* and a loading control probe (rDNA). Figure 10B shows constitutive activation of the ethylene-induced CH5B-GUS reporter gene in *ERF1*-overexpressing transgenic plants.

30 DETAILED DESCRIPTION OF THE INVENTION

WO 00/32761

PCT/US99/28104

Cascades of enzymes are a common theme in gene regulation, present in virtually all organisms from bacteria to humans, and involved in the regulation of processes as diverse as nitrogen fixation, embryogenesis, cell differentiation, response to pathogens, injury or extracellular signals, or circadian rhythmicity. The existence of a hierarchy or cascade of transcription factors and enzymes in the signaling pathway for ethylene, therefore, provides a means for regulating the complex plant response to this gaseous plant growth regulator/stress signal.

The present invention provides the cloning and characterization of *Ethylene-Response-Factor1* (*ERF1*), an early ethylene responsive gene, which encodes a GCC-box binding protein. *EIN3* expression is both necessary and sufficient for *ERF1* transcription. Like the over-expression of *EIN3* in transgenic plants, the constitutive expression of *ERF1* results in activation of a variety of ethylene response genes and phenotypes. Moreover, *EIN3* and *EILs* are demonstrated to be novel sequence-specific DNA-binding proteins that bind a primary ethylene response element in the promoter of *ERF1*. Consistent with the biochemical studies, genetic analysis revealed that *ERF1* acts downstream of *EIN3* and all previously identified components in the ethylene gas signaling pathway.

The sequential action of *EIN3* (or *EIL*) and *ERF1* DNA binding proteins adds a new level of complexity in the regulatory hierarchy of the ethylene-signaling pathway. As diagramed in Figure 1, binding of ethylene (C_2H_4) to membrane receptors activates *EIN3*, and apparently *EIL1* and *EIL2*, through a signaling cascade described elsewhere (Chao *et al.*, 1997). *EIN3* directs the expression of *ERF1* and other primary target genes by directly binding, as a dimer, to the primary ethylene response element (PERE) present in their promoters. Thus, sequences in the promoter of *ERF1* serve as an immediate target of *EIN3* binding. Although *EIN3* is sufficient and necessary for *ERF1* expression, other factors, *e.g.*, *EIL* activity, may be required for full ethylene-dependent *ERF1* induction. Consistent with this observation, *HOOKLESS1*, which contains a GCC element in its promoter, is not induced in *ERF1* transgenic plants, indicating that *ERF1* is responsible for the activation of a subset of the ethylene-responsive GCC-box-containing target genes. The subset of GCC-box-containing secondary response genes of the ethylene signaling pathway, which are activated or modulated by the *ERF1* of the present

WO 00/32761

PCT/US99/28104

invention are referred to herein simply as the "target genes" or "target secondary ethylene response genes" or genes having "the described GCC-box binding capability." Such a subset is readily identified by one of ordinary skill in the art without undue experimentation.

5 Following ethylene induction or constitutive expression, ERF1 activates the transcription of downstream target secondary ethylene response genes. ERF1 binds to the GCC box (secondary ethylene response element, SERE), thereby activating the expression of certain secondary ethylene response genes, such as basic-chitinases and defensins (*PDF1.2*). The secondary ethylene response genes are also referred to herein
10 as ethylene-inducible target genes or effector genes in the ethylene signaling pathway.

 The EREBP family of DNA-binding proteins have been identified based on their ability to bind to the GCC-box, and element that has been identified as being necessary for ethylene inducibility in response to pathogen attack as reviewed by Deikman, *Physiol. Plantarum* 100:561-566 (1997). In fact, interactions between EREBPs and bZIP
15 transcription factors, as well as synergistic effects of their DNA target sites, the GCC box and G box, have been previously described (Hart *et al.*, 1993; Sessa *et al.*, 1995; Buttner *et al.*, 1997).

 However ERF1, although a member of the ethylene response cascade, is neither anticipated nor suggested by known members of the EREBP family. For example, one
20 member of the Arabidopsis AP2/EREBP family, AtEBP, is reportedly regulated by ethylene (Buttner *et al.*, 1997), and like *ERF1*, *AtEBP* is constitutively expressed in *ctr1* mutants. However, AtEBP does not appear to be a direct target of EIN3 since its expression in response to ethylene is cycloheximide-dependent, suggesting a regulatory cascade exists among members of the EREBP family in which AtEBP acts downstream
25 of ERF1. Consistent with this idea, several of the EREBP genes were found to contain GCC box elements in their promoters, indicating that their expression is auto-regulated or controlled by other EREBPs. Thus, the existence of a transcriptional regulatory cascade is not constrained to EREBPs involved in ethylene signaling since it has been also inferred in the case of RAP genes (AP2/ EREBP family members), which are not
30 obviously involved in the response to ethylene (Okamuro *et al.*, 1997).

WO 00/32761

PCT/US99/28104

It should be appreciated that the present invention is not limited to the proposed models and mechanisms described herein. It should be further recognized that models, such as Fig. 1, present a working model merely to schematically show the role of the interactive components of the ethylene signal transduction pathway, and their relationships to ERF1, as well as to each other. Thus, the model is intended simply to facilitate understanding of the invention.

Ethylene is an olefin, therefore its receptors are presumed to require coordination of a transition metal for hormone binding activity. In wild type plants, ethylene binding to its receptor inactivates the activity of ethylene receptors (presumably causing a reduction in the histidine kinase activity), and consequently causing induction of the ethylene response through activation (de-repression) of the signaling pathway. To identify mutations in novel components of the ethylene gas signal transduction pathway, a screen was initiated for *Arabidopsis thaliana* mutants that exhibited an ethylene-like triple response phenotype in response to a potent hormone antagonist. The "triple response" in *Arabidopsis* consists of three distinct morphological changes in dark-grown seedlings upon exposure to ethylene: 1) inhibition of hypocotyl and root elongation, 2) radial swelling of the stem and 3) exaggeration of the apical hook. A class of constitutive mutants, *ctr*, display a constitutive triple response in the presence of ethylene biosynthetic inhibitors, and is affected at, or downstream of the receptor. Screening includes screening for root or stem elongation and screening for increased ethylene production.

By "plant" as used herein, is meant any plant and any part of such plant, be it wild type, treated, genetically manipulated or recombinant, including transgenic plants. The term broadly refers to any and all parts of the plant, including plant cell, tissue, flower, leaf, stem, root, organ, and the like, and also including seeds, progeny and the like, whether such part is specifically named or not. "Modified plants" are plants in which the wild-type gene or protein character has been altered. Phenotypic alterations may enhance or inhibit a typical wild-type response in or by the plant cells; or there may be no phenotypic effect whatsoever.

As one skilled in the art will recognize, absolute levels of endogenous ethylene production by a plant or plant cell will change with growth conditions. However, in

WO 00/32761

PCT/US99/28104

“ethylene sensitive” plants, including wild type plants or plants in which the signaling cascade is complete, secondary ethylene responses are activated by ERF1. Such plants or plant cells typically demonstrate a shut-down or diminution of endogenous gas production in the presence of high concentrations of exogenous ethylene.

5 By comparison, an “ethylene insensitive” plant or plant cell typically continues to produce endogenous ethylene, despite administration of inhibitory amounts of exogenous ethylene. An ethylene insensitive plant will produce more ethylene or produce it at a rate greater than that of a wild type plant upon administration of an inhibitory amount of exogenous ethylene. For the purposes of the present invention, ethylene insensitivity
10 includes either a total or partial inability to display the triple response in the presence of increased levels of exogenous ethylene, such as would be expected if the ethylene signaling pathway in were interrupted in the plant or plant cell. In such ethylene insensitive plants, the impaired receptor function resulting from blocking or inhibiting the expression of the *EIN3* (or *EIL*) or *ERF1* genes will be displayed as constitutive
15 production of endogenous ethylene, despite the presence of an abundance of exogenous ethylene.

The gene corresponding to *ERF1* has been cloned as set forth below and the sequence of the cDNA clone is provided as SEQ ID No:1. Nevertheless, in view of the descriptions provided, it is understood that other alleles and variations would be available
20 to one of ordinary skill in the art. Therefore, additional mutants are also enabled by the present invention that have insertions, deletions, alterations or substitutions within the same conserved protein motif, so long as GCC-box binding activity is expressed or regulated.

The invention should be construed to include the nucleic acid comprising *erf1*, or
25 any mutant, derivative, homologue or fragment thereof, so long as it still encodes an early ethylene responsive gene which encodes a binding element for the GCC-box, and which is capable of activating or modulating the expression of target secondary ethylene response genes in the ethylene signaling pathway. In accordance with the present invention, nucleic acid sequences include, but are not limited to DNA, including and not
30 limited to cDNA and genomic DNA; RNA, including and not limited to mRNA and tRNA, and may include chiral or mixed molecules.

WO 00/32761

PCT/US99/28104

Preferred nucleic acid sequences include, for example, the sequence set forth in SEQ ID No: 1, as well as modifications of the nucleic acid sequence, including alterations, insertions, deletions, mutations, homologues and fragments thereof encoding the active region of ERF1 or an ERF1-type regulatory protein in the ethylene response pathway capable of ERF1-GCC-box binding activity resulting in modulation of the expression of a target secondary ethylene response gene.

Expression of the secondary ethylene response genes is modulated by ERF1. "Modulation" of expression by ERF1 preferably means activation of expression, although in the present invention it is also meant to include enhanced expression of the target genes. Depending on conditions, however, it is also meant to include inhibition or prevention of expression of the target genes.

A "fragment" of a nucleic acid is included within the present invention if it encodes substantially the same expression product as the isolated nucleic acid, or if it encodes a peptide having the described GCC-box binding capability.

The invention should also be construed to include peptides, polypeptides or proteins comprising ERF1, or any mutant, derivative, variant, analogs, homologue or fragment thereof, having the described GCC-box binding capability in the ethylene signaling pathway. The terms "protein," "peptide," "polypeptide," and "protein sequences" are used interchangeably within the scope of the present invention, and include, but are not limited to the sequence set forth in SEQUENCE ID NO: 2, the putative amino acid sequences corresponding to nucleic acid SEQUENCE ID NO: 1, as well as those sequences representing mutations, derivatives, analogs or homologues or fragments thereof having the described GCC-box binding capability in the ethylene response pathway.

The invention also provides for analogs or homologues of proteins, peptides or polypeptides encoded by the gene of interest, preferably *ERF1*. "Analog" can differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both. "homologues" are chromosomal DNA carrying the same genetic loci; when carried on a diploid cell there is a copy of the homologue from each parent.

WO 00/32761

PCT/US99/28104

For example, conservative amino acid changes may be made, which although they alter the primary sequence of the peptide, do not normally alter its function.

Conservative amino acid substitutions of this type are known in the art, *e.g.*, changes within the following groups: glycine and alanine; valine, isoleucine and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; or phenylalanine and tyrosine.

Modifications (which do not normally affect the primary sequence) include *in vivo* or *in vitro* chemical derivatization of the peptide, *e.g.*, acetylation or carbonation. Also included are modifications of glycosylation, *e.g.*, modifications made to the glycosylation pattern of a polypeptide during its synthesis and processing, or further processing steps. Also included are sequences in which amino acid residues are phosphorylated, *e.g.*, phosphotyrosine, phosphoserine or phosphothreonine.

Also included in the invention are polypeptides which have been modified using ordinary molecular biology techniques to improve their resistance to proteolytic degradation or to optimize solubility or to render them more effective as a regulatory agent. Analogs of such peptides include those containing residues other than the naturally occurring L-amino acids, *e.g.*, D-amino acids or non-naturally occurring synthetic molecules. However, the polypeptides of the present invention are not intended to be limited to products of any specific exemplary process defined herein.

"Derivative" is intended to include both functional and chemical derivatives, including fragments, segments, variants or analogs of a molecule. A molecule is a "chemical derivative" of another, if it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, and the like, or they may decrease toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, and the like. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art. Included within the meaning of the term "derivatives" as used in the present invention are "alterations," "insertions," and "deletions" of nucleotides or peptides, polypeptides or the like.

WO 00/32761

PCT/US99/28104

A "fragment" of a polypeptide is included within the present invention if it retains substantially the same activity as the purified peptide, or if it has the described GCC-box binding capability. Such fragment of a peptide is also meant to define a fragment of an antibody.

5 A "variant" or "allelic or species variant" of a protein refers to a molecule substantially similar in structure and biological activity to the protein. Thus, if two molecules possess a common activity and may substitute for each other, it is intended that they are "variants," even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the amino
10 acid or nucleotide sequence is not identical.

In accordance with the invention, the ERF1 polypeptide and *ERF1* nucleic acid sequences employed in the invention may be exogenous sequences. Exogenous or heterologous, as used herein, denotes a nucleic acid sequence which is not obtained from and would not normally form a part of the genetic makeup of the plant, cell, organ,
15 flower or tissue to be transformed, in its untransformed state. Plants comprising exogenous nucleic acid sequences of *ERF1*, or *erf1* mutations are encoded by, but not limited to, the nucleic acid sequences of SEQ ID No: 1, including alterations, insertions, deletions, mutations, homologues and fragments thereof.

Transformed plant cells, tissues and the like, comprising nucleic acid sequence of
20 ERF1 or *erf1* mutations, such as, but not limited to, the nucleic acid sequence of SEQ ID No: 1 are within the scope of the invention. Transformed cells of the invention may be prepared by employing standard transformation techniques and procedures as set forth in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

25 By the term "nucleic acid encoding" the plant cell and the like wherein secondary target cell expression is modulated by GCC-box binding, as used herein is meant a gene encoding a polypeptide having the described GCC-box binding capability which activates or modulates the expression of ERF1-regulated secondary ethylene response genes. The term is meant to encompass DNA, RNA, and the like.

30 As described in the following Examples, *ERF1* genes encode proteins having specific domains located therein, for example, terminal extensions, transmembrane

WO 00/32761

PCT/US99/28104

spans, TM1 and TM2, nucleotide binding folds, a putative regulatory domain, and the C-terminus. A mutant, derivative, homologue or fragment of the subject gene is, therefore also one in which selected domains in the related protein share significant homology (at least about 40% homology) with the same domains in the preferred embodiment of the present invention. It will be appreciated that the definition of such a nucleic acid encompasses those genes having at least about 40% homology, in any of the described domains contained therein under conditions of stringency that would be appreciated by one of ordinary skill in the art.

In addition, when the term "homology" is used herein to refer to the domains of these proteins, it should be construed to mean homology at both the nucleic acid and the amino acid levels. Significant homology between similar domains in such nucleic acids is considered to be at least about 40%, preferably, the homology between nucleic acid domains is at least about 50%, more preferably, at least about 60%, even more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% and most preferably, the homology between similar nucleic acid domains is about 99%. Significant homology between similar amino acid domains in such protein or polypeptides is considered to be at least about 40%, preferably, the homology between amino acid domains is at least about 50%, more preferably, at least about 60%, even more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% and most preferably, the homology between similar amino acid domains is about 99%.

According to the present invention, preferably the isolated nucleic acid encoding the biologically active ERF1 polypeptide or fragment thereof is full length or of sufficient length to encode a regulated or active GCC-box binding protein capable of activating or modulating the expression of the secondary ethylene response genes. In one embodiment the nucleic acid is at least about 200 nucleotides in length. More preferably, it is at least 400 nucleotides, even more preferably, at least 600 nucleotides, yet more preferably, at least 800 nucleotides, and even more preferably, at least 1000 nucleotides in length. In another embodiment, preferably, the purified preparation of the isolated polypeptide having the described GCC-box binding activity in the ethylene signal system is at least about 60 amino acids in length. More preferably, it is at least 120 amino acids,

WO 00/32761

PCT/US99/28104

even more preferably, at least 300 amino acids, yet more preferably, at least 500 amino acids, and even more preferably, at least 700 amino acids in length. In an additional embodiment the polypeptide encodes the full-length ERF1 protein or a regulated version thereof.

5 The invention further includes a vector comprising a gene encoding ERF1. DNA molecules composed of a protein gene or a portion thereof, can be operably linked into an expression vector and introduced into a host cell to enable the expression of these proteins by that cell. Alternatively, a protein may be cloned in viral hosts by introducing the Ahybrid® gene operably linked to a promoter into the viral genome. The protein may
10 then be expressed by replicating such a recombinant virus in a susceptible host. A DNA sequence encoding a protein molecule may be recombined with vector DNA in accordance with conventional techniques. When expressing the protein molecule in a virus, the hybrid gene may be introduced into the viral genome by techniques well known in the art. Thus, the present invention encompasses the expression of the desired proteins
15 in either prokaryotic or eukaryotic cells, or viruses that replicate in prokaryotic or eukaryotic cells.

Preferably, the proteins of the present invention are cloned and expressed in a virus. Viral hosts for expression of the proteins of the present invention include viral particles which replicate in prokaryotic host or viral particles which infect and replicate
20 in eukaryotic hosts. Procedures for generating a vector for delivering the isolated nucleic acid or a fragment thereof, are well known, and are described for example in Sambrook *et al.*, *supra*. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor inducing (Ti) plasmids (*e.g.*, pBIN19) containing a target gene under the control of a vector, such as the cauliflower mosaic (CaMV) 35S promoter (Lagrimini *et al.*, *Plant*
25 *Cell* 2:7-18 (1990)) or its endogenous promoter (Bevan, *Nucl. Acids Res.* 12:8711-8721(1984)), adenovirus, bovine papilloma virus, simian virus, tobacco mosaic virus and the like.

Once the vector or DNA sequence containing the constructs has been prepared for expression, the DNA constructs may be introduced or transformed into an appropriate
30 host. Various techniques may be employed, such as protoplast fusion, calcium phosphate precipitation, electroporation, or other conventional techniques. As is well known, viral

WO 00/32761

PCT/US99/28104

sequences containing the Ahybrid® protein gene may be directly transformed into a susceptible host or first packaged into a viral particle and then introduced into a susceptible host by infection. After the cells have been transformed with the recombinant DNA (or RNA) molecule, or the virus or its genetic sequence is introduced
5 into a susceptible host, the cells are grown in media and screened for appropriate activities. Expression of the sequence results in the production of the protein of the present invention.

Procedures for generating a plant cell, tissue, flower, organ or a fragment thereof, are well known in the art, and are described, for example, in Sambrook *et al.*, *supra*.
10 Suitable cells include, but are not limited to, cells from yeast, bacteria, mammal, baculovirus-infected insect, and plants, with applications either *in vivo*, or in tissue culture. Also included are plant cells transformed with the gene of interest for the purpose of producing cells and regenerating plants having the described GCC-box binding capability, thereby modulating the expression of the target secondary ethylene
15 response elements.

Suitable vector and plant combinations will be readily apparent to those skilled in the art and can be found, for example, in Maliga *et al.*, 1994, Methods in Plant Molecular Biology: A Laboratory Manual, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using *Agrobacterium*-mediated
20 leaf disc transformation methods described by Horsch *et al.*, 1988, Leaf Disc Transformation: Plant Molecular Biology Manual A5:1). Numerous procedures are known in the art to assess whether a transgenic plant comprises the desired DNA, and need not be reiterated.

The expression of the desired protein in eukaryotic hosts requires the use of
25 eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, but are not limited to, the SV40 early promoter (Benoist *et al.*, *Nature (London)* 290:304-310 (1981)); the yeast *gal4* gene promoter (Johnston *et al.*, *Proc. Natl. Acad. Sci. (USA)* 79:6971-6975 (1982)) and the exemplified pYES3 PGK1 promoter. As is
30 widely known, translation of eukaryotic mRNA is initiated at the codon, which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a

WO 00/32761

PCT/US99/28104

eukaryotic promoter and a DNA sequence which encodes the desired protein does not contain any intervening codons which are capable of encoding a methionine (*i.e.*, AUG).

The desired protein encoding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the desired protein may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome. For expression of the desired protein in a virus, the hybrid gene operably linked to a promoter is typically integrated into the viral genome, be it RNA or DNA. Cloning into viruses is well known and thus, one of skill in the art will know numerous techniques to accomplish such cloning.

Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more reporter genes or markers which allow for selection of host cells which contain the expression vector. The reporter gene or marker may complement an auxotrophy in the host (such as *leu2*, or *ura3*, which are common yeast auxotrophic markers), biocide resistance, e.g., antibiotics, or resistance to heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, H., *Mol. Cell. Biol.* 3:280 (1983), and others.

In another embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host cell. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a

WO 00/32761

PCT/US99/28104

particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

The invention further defines methods for manipulating the nucleic acid in a plant to permit the regulation, control or modulation of germination, abscission, cell
5 elongation, sex determination, flower or leaf senescence, flower maturation, fruit ripening, insect, herbicide or pathogen resistance, or response to stress in said plant. In a preferred embodiment the method activates or enhances the above responses, whereas in another preferred embodiment the method inhibits or prevents the above responses.

Thus, methods of the present invention define embodiments in which the GCC-
10 box binding activity is prevented or inhibited. By "prevention" is meant the cessation of GCC-box binding to target secondary response proteins in the ethylene signal pathway. By "inhibition" is meant a statistically significant reduction in the amount of GCC-box binding binding, or in the amount of expression of the target ethylene response cells, or of detectable ERF1 protein as compared with plants, plant cells, organs, flowers or
15 tissues grown without the inhibitor or disclosed method of inhibition. Preferably, by blocking or inhibiting ERF1 the ERF1 inhibitor reduces GCC-box binding thereby inhibiting or reducing expression of the secondary ethylene response gene by at least 20 %, more preferably by at least 50%, even more preferably by 80% or greater, and also preferably, in a dose-dependent manner. The effect of such prevention or inhibition
20 would block (insensitivity) or inhibit the ethylene response of a plant or plant cell comprising such DNA or protein expression product.

In accordance with another preferred embodiment of the invention, once inhibitors satisfying these requirements are identified, the utilization of assay procedures to identify the manner in which the GCC-box binding or target gene expression is
25 inhibited are particularly useful.

Ethylene insensitive plants are disease and pathogen tolerant. For purposes of the present invention, disease tolerance is the ability of a plant or plant cell to survive stress, infection or injury with minimal damage or reduction in the harvested yield of commercial product. Plants with disease tolerance may have extensive levels of
30 infection, but little necrosis and few or no lesions. The plants or plant cells may also have reduced necrotic and water soaking responses and chlorophyll loss may be virtually

WO 00/32761

PCT/US99/28104

absent. In contrast, resistant plants generally limit the growth of the pathogens and contain the infection to a localized area within multiple apparently injurious lesions.

Similarly, methods of the present invention are also defined in which the GCC-box binding activity is initiated, stimulated or enhanced if there is a statistically
5 significant increase in the amount of GCC-box binding or in the amount of expression of the target ethylene response cells, or of detectable ERF1 protein detected as compared with plants, plant cells, organs, flowers or tissues grown without the enhancer or disclosed method of enhancement. Preferably, the ERF1 enhancer increases binding capability by at least 20 %, more preferably by at least 50%, even more preferably by
10 80% or greater, and also preferably, in a dose-dependent manner. Once enhancers satisfying these requirements are identified, the utilization of assay procedures to identify the manner in which the GCC-box binding is enhanced are particularly useful.

The invention further features an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of *ERF1* or of a gene encoding a peptide
15 having plant GCC-box binding capability. The antisense nucleic acid should be of sufficient length as to inhibit expression of the target gene of interest. The actual length of the nucleic acid may vary, depending on the target gene, and the region targeted within the gene. Typically, such a preparation will be at least about 15 contiguous nucleotides, more typically at least 50 or even more than 50 contiguous nucleotides in length.

20 As used herein, a sequence of nucleic acid is considered to be antisense when the sequence being expressed is complementary to, and essentially identical to the non-coding DNA strand of the *ERF1* gene, but which does not encode ERF1.

"Complementary" refers to the subunit complementarity between two nucleic acids, *e.g.*, two DNA molecules. When a nucleotide position in both molecules is occupied by
25 nucleotides normally capable of base pairing with each other, then the nucleic acids are said to be complementary to each other. Thus two nucleic acids are complementary when a substantial number (at least 40% or at least 50%, or preferably at least 60%, or more preferably at least 70% or 80%, or more preferably at least 90% or at least 99%) of the corresponding positions in each of the molecules are occupied by nucleotides which
30 normally base-pair with each other (*e.g.*, A:T and G:C nucleotide pairs.).

WO 00/32761

PCT/US99/28104

In yet another aspect of the invention, antibodies are provided which are directed against the GCC-box binding peptides or polypeptides, such as ERF1, which are capable of binding the GCC-box of secondary response proteins in the ethylene signaling pathway, thereby blocking or modulating their expression. Such an antibody is specific
5 for the whole molecule, its N- or C-terminal, or internal portions. Methods of generating such antibodies are well known in the art.

In the embodiment directed to the antibody specific for a plant ERF1 polypeptide, including functional equivalents of the antibody, the term "functional equivalent" refers to any molecule capable of specifically binding to the same antigenic determinant as the
10 antibody, thereby neutralizing the molecule, *e.g.*, antibody-like molecules, such as single chain antigen binding molecules.

The invention further includes a transgenic plant comprising an isolated DNA encoded ERF1 or a GCC-box binding protein capable of activating the expression of secondary ethylene response genes in the plant ethylene signaling pathway. For instance
15 provided in at least one example of the current invention, a transgenic *Arabidopsis* plant comprising a yeast *cccΔ* transgene rescued by the addition of *ERF1*, which when expressed confers upon the plant the ability recognize the presence of ethylene, an ability that had been deleted from the original yeast gene.

By "transgenic plant" as used herein, is meant a plant, plant cell, tissue, flower,
20 organ, including seeds, progeny and the like, or any part of a plant, which comprise a gene inserted therein, which gene has been manipulated to be inserted into the plant cell by recombinant DNA technology. The manipulated gene is designated a "transgene." The "non-transgenic," but substantially homozygous "wild type plant" as used herein, means a nontransgenic plant from which the transgenic plant was generated. The
25 transgenic transcription product may also be oriented in an antisense direction as describe above.

The generation of transgenic plants comprising sense or antisense DNA encoding the GCC-box binding molecules, such as ERF1, capable of activating, blocking or modulating the expression ethylene-inducible target genes, may be accomplished by
30 transforming the plant with a plasmid, liposome, or other vector encoding the desired DNA sequence. Such vectors would, as described above, include, but are not limited to

WO 00/32761

PCT/US99/28104

the disarmed *Agrobacterium* tumor-inducing (Ti) plasmids containing a sense or antisense strand placed under the control of a strong constitutive promoter, such as the CaMV 35S promoter or under an inducible promoter. Methods of generating such constructs, plant transformation and plant regeneration methods are well known in the art
5 once the sequence of the gene of interest is known, for example as described in Ausubel
et al., 1993, *Current Protocols in Molecular Biology*, Greene & Wiley, New York).

In accordance with the present invention, plants included within its scope include both higher and lower plants of the Plant Kingdom. Mature plants, including rosette stage plants, and seedlings are included in the scope of the invention. A mature plant,
10 therefore, includes a plant at any stage in development beyond the seedling. A seedling is a very young, immature plant in the early stages of development.

Transgenic plants are also included within the scope of the present invention, having a phenotype characterized by the *ERF1* gene or *erf1* mutations, or by the *EIN3* gene or *ein3* mutations (including *eil* mutations) affecting the activation of, or expression
15 of, the RTF3.

Preferred plants of the present invention, which are affected by ERF1 or GCC-box binding to modulate expression of the secondary ethylene response genes in the ethylene signal system include, but are not limited to, high yield crop species for which cultivation practices have already been perfected (including monocots and dicots, *e.g.*,
20 alfalfa, cashew, cotton, peanut, fava bean, french bean, mung bean, pea, walnut, maize, petunia, potato, sugar beet, tobacco, oats, wheat, barley and the like), or engineered endemic species. Particularly preferred plants are those from: the Family *Umbelliferae*, particularly of the genera *Daucus* (particularly the species *carota*, carrot) and *Apium* (particularly the species *graveolens dulce*, celery) and the like; the Family *Solanaceae*,
25 particularly of the genus *Lycopersicon*, particularly the species *esculentum* (tomato) and the genus *Solanum*, particularly the species *tuberosum* (potato) and *melongena* (eggplant), and the like, and the genus *Capsicum*, particularly the species *annuum* (pepper) and the like; and the Family *Leguminosae*, particularly the genus *Glycine*, particularly the species *max* (soybean) and the like; and the Family *Cruciferae*, particularly of the genus
30 *Brassica*, particularly the species *campestris* (turnip), *oleracea* cv Tasti (cabbage), *oleracea* cv Snowball Y (cauliflower) and *oleracea* cv Emperor (broccoli) and the like;

WO 00/32761

PCT/US99/28104

the Family *Compositae*, particularly the genus *Lactuca*, and the species *satira* (lettuce), and the genus *Arabidopsis*, particularly the species *thaliana* (Thale cress) and the like. Of these Families, the more preferred are the leafy vegetables, for example, the Family *Cruciferae*, especially the genus *Arabidopsis*, most especially the species *thaliana*.

5 Preferred plants particularly include flowering plants, such as roses, carnations, chrysanthemums, geraniums and the like, in which longevity of the flower on the stem (delayed abscission) is of particular relevance, and especially include ornamental flowering plants, such as geraniums. Additional preferred plants include leafy green ornamental plants, such as *Ficus*, palms, and the like, in which longevity of the leaf stem
10 on the plant (delayed abscission) is of particular relevance. Delayed flowering in such plants may also be advantageous. Similarly, other preferred plants include fruiting plants, such as banana and orange, wherein pectin-dissolving enzymes are involved in the abscission process.

The present invention will benefit plants subjected to stress. Stress includes, and
15 is not limited to, infection as a result of pathogens such as bacteria, viruses, fungi, and conditions involving aging, wound healing and soil penetration. Bacterial infections include, and are not limited to, *Clavibacter michiganense* (formerly *Corynebacterium michiganense*), *Pseudomonas solanacearum* and *Erwinia stewartii*, and more particularly, *Xanthomonas campestris* (specifically pathovars *campestris* and
20 *vesicatoria*), *Pseudomonas syringae* (specifically pathovars tomato, *maculicola*).

In addition to bacterial infections, other examples of plant viral and fungal pathogens within the scope of the invention include, but are not limited to, tobacco mosaic virus, cauliflower mosaic virus, turnip crinkle virus, turnip yellow mosaic virus; fungi including *Phytophthora infestans*, *Peronospora parasitica*, *Rhizoctonia solani*,
25 *Botrytis cinerea*, *Phoma lingam* (*Leptosphaeria maculans*), and *Albugo candida*.

Through continued isolation of mutants of the cascade of enzymes in the plant ethylene signaling pathway it will be possible, in accordance with the present invention, to identify additional genes required for assembly of functional ethylene receptors, and for use as control mechanisms of the plant response to this gaseous hormonal maturation,
30 stress or pathogen regulator.

WO 00/32761

PCT/US99/28104

The present invention is further described in the following examples. These examples are not to be construed as limiting the scope of the appended claims.

EXAMPLES

5 Strains and Growth Conditions

The *Arabidopsis* ecotype Columbia (Col-0) was the parent strain of all mutants and transgenic plants used in the following examples. Triple response screens were performed as described previously by Guzman and Ecker, *Plant Cell* 2:513-523 (1990). Plant growth in air and ethylene was carried out as described previously by Kieber *et al.*,
10 1993.

Nucleic Acid Analysis

Total RNA extractions and Northern analyses were performed as described by Reuber *et al.*, *Plant Cell* 8:241-249 (1996) and by Chao *et al.*, 1997. β -glucuronidase activity was assayed by incubation of the plants with the substrate of the enzyme (X-
15 Gluc., 1 mg/ml) in sodium phosphate buffer during 18 h.. cDNA clones corresponding to *ERF1* were isolated by hybridization of a size-selected cDNA library in λ ZAPII (Kieber *et al.*, 1993). The probe, corresponding to a fragment of the tobacco *EREBP1* gene, was obtained by PCR amplification using the primers:

EREBP1f: 5' CACGCCATAGACATAATAC 3' (SEQ ID No: 3) and
20 *EREBP1r*: 5' GCTACGATTCCTGTTCTTCAG 3' (SEQ ID No: 4).

ERF1 genomic sequences were isolated by hybridization of two BAC genomic libraries (TAMU and IGF) (Choi *et al.*, *Weeds World* 2:17-20 (1995)). The map position of *ERF1* was obtained by PCR amplification of YAC pools using specific primers. PCR highlighted two YAC clones (CIC12H5 and CIC12H6), both located in the ABI3 contig.

25 **Example 1: Characterization of the *ERF1* gene and its Expression Product**

Protein synthesis and DNA analysis

Full length *ERF1*, *EIN3* and the *EIN3* deletion derivatives were generated by *in vitro* translation (or cotranslation in the dimerization experiments) using a flexi-rabbit reticulocyte lysate system (Promega™) as described by Solano *et al.*, *J. Biol. Chem.*
30 272:2889-2895 (1997). PCR and Klenow labeling of promoter fragments and oligonucleotides, DNA binding reactions and electrophoretic mobility shift assays

WO 00/32761

PCT/US99/28104

(EMSAs) were performed as described Solano *et al.*, *EMBO J.* 14:1773-1784 (1995).

The promoter fragments of the *Arabidopsis* and bean basic-chitinase (*CHI*) genes containing the GCC box were obtained by Klenow filling of the following overlapping primers:

5 b-CHIforward: 5' GTTGATCACGAACCCGCCGCTCATATTCATAATTA 3'
(SEQ ID No: 5);

 b-CHImutant: 5' GTTGATCACGAACCCGTTGTTTCATATTCATAATTA 3'
(SEQ ID No: 6);

 b-CHIreverse: 5' TTTAACTTTAATTATGAATATGA 3' (SEQ ID No: 7);

10 CH5Bforward: 5' CTTACAGCTTGGGAAGCCGCCGGGGTGGGCCCCGCAG
3' (SEQ ID No: 8);

 CH5Bmutant: 5' CTTACAGCTTGGGAAGTTGTTGGGGTGGGCCCCGCAG 3'
(SEQ ID No: 9); and

 CH5Breverse: 5' AAACCTTTCTGCGGGCCCCACCCC 3' (SEQ ID No: 10).

15 Sequences of the mutant versions of EBS used in competition experiments are:

 EBSm1: 5' GTTGTTTGGGATTCTTCGGGCATGTATCTTGAATCC 3' (SEQ
ID No: 11) and

 EBSm2: 5' GTTGTTTGGGATTCAAGCCCCATGTATCTTGAATCC 3' (SEQ
ID No: 12).

20 Plant transformation

 A 0.8 kb BamHI-KpnI fragment of *ERF1* cDNA was cloned into BamHI-KpnI-digested pROK2 (Baulcombe, *Nature* 321:446-449 (1983)). The C58 strain of *Agrobacterium tumefaciens* containing the above construct was used to transform the *Arabidopsis* ecotype Col-0 and the ethylene insensitive mutants *ein2-5*, *ein2-17*, *ein2-26*,
25 *ein3-1*, *ein3-3* and *ein5-1*, by *in planta* vacuum infiltration (Bechtold *et al.*, *C. R. Acad. Sci. Paris Life Sci.* 316:1194-1199 (1993). Kanamycin resistant (kan^R) T1 plants were selected by plating seeds on MS medium supplemented with 100 $\mu\text{g/ml}$ kanamycin, and transferring kan^R seedlings to soil.

Yeast transformation and "two-hybrid" screening

30 Yeast strain Y190 was transformed by the PEG/lithium acetate method as described by Gietz *et al.*, *Nucleic Acid. Res.* 20:1425 (1992). Growth conditions,

WO 00/32761

PCT/US99/28104

screening procedures and filter-lift assay for β -galactosidase activity were performed as described by Kim *et al.*, *Proc. Natl. Acad. Sci. USA* 94:11786-11791 (1997).

GenBank Accession Numbers

GenBank accession numbers for the *ERF1* cDNA and genomic sequences
5 identified in the present invention are AF076277 and AF076278, respectively.

Cloning and characterization of *ERF1*

To identify targets of the EIN3/EIL proteins and to examine the role of EREBPs
in the ethylene signaling pathway, a PCR-based approach was used to isolate members of
the EREBP family in *Arabidopsis*. Using oligonucleotides complementary to the tobacco
10 EREBP1 sequence (Ohme-Takagi *et al.*, 1995), a 597 bp fragment was amplified from
tobacco genomic DNA, and this fragment was used to screen an *Arabidopsis* cDNA
library under low stringency.

Among the resulting positive clones, two classes of cDNAs showed high
homology to the tobacco *EREBP1/2* and *EREBP3/4* genes. Total RNA was isolated from
15 4 weeks-old Wt Col-0 (W) or *ein3-1* (M) plants grown in air and exposed to ethylene gas
for different times (0 to 48 hours). Thirty μ g of total RNA was loaded per lane in Figure
2A and 2C, and 60 μ g/lane in Figure 2B.

One gene, called *Ethylene-Response-Factor1* (*ERF1*) showed rapid induction in
response to ethylene. More importantly, *ERF1* mRNA began to accumulate after 15
20 minutes of hormone treatment of plants. Induction of *ERF1* mRNA was also dependent
on the presence of functional EIN3 as shown in Figure 2A.

In order to compare the kinetics of *ERF1* induction with that of a known
ethylene-inducible gene, the same blot was hybridized with *PDF1.2*, a member of the
defensin gene family (Penninckx *et al.*, 1996). As expected if *ERF1* is a regulator of
25 these genes, maximal *ERF1* expression occurred earlier than *PDF1.2* (Figure 2A).

Since overexpression of the ethylene pathway genes *EIN3*, *EIL1* or *EIL2* causes
activation of all known ethylene response genes and phenotypes, the expression of *ERF1*
was examined. Although the level was somewhat lower than that achieved by exogenous
ethylene treatment, *ERF1* mRNA showed constitutive high-level expression in
30 35S::*EIN3*-expressing transgenic plants (see Figure 2B), indicating that EIN3 is
sufficient for *ERF1* expression. Taken together, the results demonstrate that *EIN3* is both

WO 00/32761

PCT/US99/28104

necessary and sufficient for expression of the early ethylene response gene *ERF1*, a novel AP2/EREBP-type DNA binding protein

To confirm that *ERF1* is a primary ethylene response gene, induction by the hormone's dependence on protein synthesis was tested using cycloheximide as described
5 by Lam *et al.*, *EMBO J.* 8:2777-2783 (1989) and Abel *et al.*, *J. Mol. Biol.* 251:533-549 (1995). Cycloheximide treatment was found to induce the expression of *ERF1* at least 20 to 50 fold higher than that observed by ethylene treatment, masking the ethylene inducibility (Figure 2C).

ERF1 was mapped to chromosome III, in the ABI3 contig, by PCR amplification
10 of YAC pools using *ERF1*-specific primers. Other than *EIN3*, none of the known ethylene signaling mutants mapped to this region.

Example 2: ERF1 is Downstream Component in Ethylene Gas Signaling Pathway

Previous efforts to understand the hormonal regulation of ethylene-regulated
15 genes in several plant systems led to the identification of two kinds of ethylene response elements (EREs). One type of ERE was found to be responsible for ethylene-regulated expression of genes induced during senescence (Itzhaki *et al.*, 1994). A second element, the "GCC box", was identified as being necessary for ethylene inducibility in response to pathogen attack (reviewed by Deikman, 1997). Based on an ability to bind to the GCC
20 element, a family of DNA-binding proteins (EREBPs) was identified in tobacco (Ohme-Takagi *et al.*, 1995). The fact that these genes were themselves transcriptionally activated by treatment with ethylene suggested that they act as an intermediate step between the EIN3/EIL proteins and downstream effector genes, such as basic-chitinase.

To identify targets of EIN3, members of the *Arabidopsis* EREBP family were
25 cloned and characterized. *ERF1* was rapidly induced in response to ethylene gas and constitutively expressed in the presence of the ethylene pathway mutant *ctr1*. Ethylene induction of *ERF1* was completely dependent on a functional EIN3 protein, since no expression was detected in the *ein3-1* mutant. Moreover, transgenic plants overexpressing EIN3 showed high-level expression of *ERF1* mRNA.

30 The results indicate that EIN3 is both necessary and sufficient for *ERF1* expression, conclusions which are consistent with *ERF1* being a direct target of EIN3.

WO 00/32761

PCT/US99/28104

The level of *ERF1* mRNA expression in EIN3 overexpressing plants was somewhat lower than in *ctr1* mutants or in ethylene treated wild-type plants. This indicated that although EIN3 is sufficient for *ERF1* expression, other factors are required for full ethylene-dependent *ERF1* induction.

5 Using EIN3 as a "bait" in the "two-hybrid" screen, a DNA-binding protein was identified that interacts with EIN3. This protein also bound to the *ERF1* promoter in a sequence specific-manner, indicating its role as a partner of EIN3 and suggesting its importance to full *ERF1* expression in response to ethylene.

 Loss-of-function mutations have not been reported for any member of the EREBP
10 family. This finding, together with the fact that over 30 of these genes have been identified in *Arabidopsis*, suggested a functional redundancy among members of the EREBP family. In the case of functionally redundant genes, loss-of-function alleles may not show a phenotype. A clear example of this has been provided by studies of the ethylene receptors in *Arabidopsis* (Hua and Meyerowitz, *Cell* (1998)). Implication in
15 the ethylene signaling pathway of each of the five *ETR1*-related genes was made through the identification (or creation by site-specific mutagenesis) of dominant mutations (Chang *et al.*, *Science* 262, 539-544 (1993); Hua *et al.*, 1995; Hua *et al.*, *Plant Cell*, 1998; Sakai *et al.*, 1998). While single loss-of-function mutations in these genes do not exhibit defects in ethylene response, triple and quadruple mutants display constitutive
20 ethylene response phenotypes, revealing that ethylene responses are negatively regulated by the receptors (Hua and Meyerowitz, *Cell*, 1998).

 For this reason, a gain-of-function strategy was used to address the *in vivo* function of *ERF1*. Gain-of-function mutations obtained by insertional mutagenesis of T-DNA or transposon elements carrying a CaMV 35S promoter (enhancer-trap/gene-trap)
25 have proven to be a powerful tool for assessing the *in vivo* function of a gene. Constitutive *ERF1* expression resulted in seedling and adult phenotypes very similar to those displayed by loss-of-function *ctr1* mutants, plants overexpressing EIN3 or EIL1 or plants grown in ethylene.

 Some significant differences were, however, observed between EIN3 and *ERF1*
30 overexpressing plants. While *ERF1* overexpression caused inhibition of hypocotyl and root cell elongation, seedlings lacked an exaggerated apical hook. Consistent with this

WO 00/32761

PCT/US99/28104

observation, *HOOKLESS1* was not induced in *ERF1* transgenic plants. Other EREBP family members appear to be responsible for activation of these target genes.

Indeed, a transposon-induced gain-of-function mutant (*tiny*) that constitutively expresses an EREBP displayed seedling phenotypes reminiscent of a partial ethylene response (Wilson *et al.*, 1996), suggesting that TINY may be a partner of ERF1 in ethylene signaling. Alternatively, ERF1 may act in concert with other enzymes in the activation of some promoters.

Example 3: Sequence-specific binding of EIN3 in the promoter of *ERF1*

To test whether the nuclear protein EIN3 is capable of DNA-binding, electrophoretic mobility shift assays (EMSA) using *in vitro* translated EIN3 protein and the 5' promoter region of the *ERF1* gene were performed. A 6 kb fragment containing the ERF1 promoter was isolated from genomic sequences (BAC F11F14) and subcloned into pBluescriptTM. Five overlapping fragments that covered approximately 1.4 kb upstream of the *ERF1* translation initiation site were amplified and radioactively labeled by PCR. As shown in Figure 3A, a slower migrating band was observed when one of the fragments (-1238 to -950) was incubated with reticulocyte lysates containing EIN3. The binding of EIN3 to the -1238 to -950 fragment was not competed by a 500-fold excess of poly-(dIdC) or -(dAdT), demonstrating the specificity of the EIN3-DNA interaction. The specific retarded band was reproducibly more intensely in lanes competed with dAdT than with dIdC, suggesting that the EIN3 target sequence may be GC-rich.

To further delimit the EIN3 target site, this fragment was subdivided and each subfragment was subjected to binding experiments. Only one of the sub-fragments (-1213 to -1179) was specifically recognized by EIN3, further confirming its sequence specificity (Figure 3B).

To demonstrate that EIN3 was in fact the protein present in the mobility-shifted band, a series of truncated EIN3 derivatives was generated and subjected to binding and EMSA using the 36 bp EIN3-binding fragment. A mobility shift that correlated with the molecular weight of each of the truncated proteins was observed, confirming the presence of EIN3 in the protein-DNA complex (Figure 3B). The smallest protein that

WO 00/32761

PCT/US99/28104

retained DNA-binding capacity was EIN3 Δ 269, delimiting the EIN3-DNA-binding domain to amino acids 1 through 359.

5 In addition, a mutant version of EIN3 that contained the amino acid substitution encoded by the *ein3-3* allele (Lys²⁴⁵ to Asn) was generated by *in vitro* translation of the corresponding mRNA. The amino acid substitution in the *ein3-3* mutant lay within the basic domain III of the EIN3 protein. Interestingly, the mutant EIN3-3 protein was unable to recognize the 36 bp target site (Figure 3B).

10 Additional evidence that EIN3 is the protein in the complex with DNA was obtained by competition of the EIN3-DNA-binding using an anti-EIN3 antibody. No mobility shift was observed in the binding reaction mixtures that included the anti-EIN3 antibody. In contrast, addition of preimmune serum had no effect on the gel shift assay (Figure 3C). Immunoblotting of the bandshift gel using anti-EIN3 antibodies further identified EIN3 as the protein in the retarded band, since the antibodies highlighted a band with the same mobility as the slow migrating complex.

15 In EMSA experiments where short DNA molecules were used, the full-length EIN3 protein produced two mobility shifted bands, whereas its deletion-derivatives retarded only one band. Since several subproducts were obtained in the EIN3 *in vitro* translation reaction, the upper band apparently corresponded to full-length EIN3 and the lower band to a truncated EIN3 derivative.

20 Additionally, further experiments demonstrated that the source of the EIN3 protein does not affect its ability to bind DNA. As with the *in vitro* translated protein, *Baculovirus*-expressed EIN3 also recognized the 36 bp fragment containing the target sequence. Moreover, in this case only one major mobility shifted band was observed in the EMSA.

25 To more precisely define the sequence requirements of EIN3-binding, scanning mutagenesis of the 36 bp fragment was performed. As seen in Figures 4A and 4B all mutations affecting the affinity of EIN3 for its target site reside within a 28 bp sequence that included two palindromic repeats separated by a central core sequence. Single mutations within the core sequence completely abolished binding, indicating that this
30 sequence is necessary for EIN3 recognition. Mutations that affected both palindromic

WO 00/32761

PCT/US99/28104

repeats greatly reduced EIN3 binding, confirming that they are also important determinants of the interaction.

Interestingly, the EIN3 binding site shows significant similarity to sequences present in the promoter regions required for ethylene responsiveness in the tomato *E4* (Montgomery *et al.*, *Proc. Natl. Acad. Sci. USA* 90:5939-5943 (1993)) and *LEACO1* genes (Blume *et al.*, *Plant J.* 12:731-746 (1997)), and in the carnation *GST1* gene (Itzhaki *et al.*, *Proc. Natl. Acad. Sci. USA* 91:8925-8929 (1994)), (Figure 4B). In *GST1*, a 197 bp promoter fragment containing this sequence was also sufficient to confer ethylene responsiveness to a minimal CaMV 35S promoter in transient assays (Itzhaki *et al.*, 1994).

To further examine the specificity of the binding to its target site, competition experiments were performed using an excess of unlabeled EIN3 Binding Site (EBS), or two mutated versions (EBSm1 and EBSm2) not recognized by EIN3. See FIGs. 5A and 5B. Each lane contains 1 nanogram of labeled EBS. As shown in Figure 5A, the formation of the EIN3-EBS complex was more efficiently competed by an excess of unlabelled EBS than by any of the EBS mutant versions, further supporting the finding that the EIN3-EBS interaction is sequence specific. Figure 5B represents a summary of EIN3 structural features and mutants used in EMSA experiments, as adapted from Chao *et al.*, 1997.

20

Example 4: EIN3 is a Novel DNA-Binding Protein that Regulates Expression of *ERF1*

Although EIN3 and EIL proteins do not share similarity with any known proteins, their nuclear location, presence of conserved basic domains and acidic regions suitable as binding and activation domains, respectively, characterize their role as transcription factors (Chao *et al.*, 1997). DNA-binding assays using *in vitro* translated and *Baculovirus*-expressed EIN3 protein demonstrated that EIN3 binds to specific sequences in the *ERF1* promoter. EMSA experiments using truncated forms of the protein or antibodies against EIN3 confirmed the presence of EIN3 in the DNA-protein complex. On the other hand, a mutant protein corresponding to the *ein3-3* allele of *EIN3* was

30

WO 00/32761

PCT/US99/28104

unable to recognize the target sequence. This mutation consists of a Lys to Asn substitution in the basic domain III, which may form part of the DNA-binding motif.

Two additional proteins that belong to the EIN3/EIL family, EIL1 and EIL2, were also able to specifically recognize the EIN3 target in the promoter of *ERF1*. Consistent with this result, EIN3 can be functionally replaced by EIL1 or EIL2 since overexpression of either of these genes in transgenic plants can complement the *ein3-1* mutation (Chao *et al.*, 1997). Deletion analysis of EIN3 permitted confirmation that its DNA-binding domain falls within the N-terminal half of the protein. This region is the most conserved among all four members of the family and does not contain any previously known DNA-binding motif. The EIN3/EIL DNA-binding domain will be further characterized by structural analysis of the proteins. Nevertheless, these four proteins (EIN3, EIL1, EIL2 and EIL3), along with a fifth more recently identified homolog (EIL4), possess several predicted α -helices, two of them rich in basic amino acids offering a DNA-interaction surface.

Scanning mutagenesis of the DNA fragment containing the target site permitted determination of the sequence requirements for the EIN3/EIL interaction. The defined target site included two inverted repeats and is recognized by the protein as a dimer. Interestingly, the EIN3 binding site shares significant identity with sequences within the promoter region of the carnation *GST1* gene that has been defined as necessary and sufficient for ethylene responsiveness. The conserved sequences are also present in the promoter regions required for ethylene responsiveness in the tomato *E4* and *LEACO1* genes. This indicates that the EIN3 target site represents a primary ethylene response element (PERE) conserved in different species where there are also orthologs of EIN3.

Consistent with this determination, one of the genes containing this element (*E4*) has been previously identified as a primary ethylene response gene (Lincoln *et al.*, *Proc. Natl. Acad. Sci. USA* 84:2793-2797 (1987)). The GCC element appears to be a secondary ethylene response element (SERE) present only in a subset of the ethylene-regulated genes (*e.g.*, pathogenesis-related genes, *HOOKLESS1* and some *EREBPs*) that are regulated by a subgroup of the EREBP family of proteins.

30

Example 5: EIN3 Recognizes its Target as a Homodimer

The presence of palindromic repeats in the EIN3 target site suggested that EIN3 would interact with its target as a dimer. To address this question, using the approach of Hope *et al.*, *EMBO J.* 6:2781-2784 (1987), full length EIN3 and several carboxyl terminal deletion derivatives were translated *in vitro*, alone or in pair-wise combinations. The resulting translation or co-translation products were tested for DNA binding to the EBS. As shown in Figure 6A, in addition to the bands corresponding to the full size EIN3 and deletion derivatives bound to DNA, a band of intermediate mobility appeared when the co-translation products were used. The intermediate band corresponds to the mobility shift for a heterodimer, confirming that these proteins bind to the EBS as dimers.

Additional evidence that EIN3 has the capacity to form dimers came from screening for EIN3-interacting proteins using the yeast "two-hybrid" system (Fields *et al.*, *Nature* 340:245-246 (1989); Durfee *et al.*, *Genes Dev.* 7:555-569 (1993)). Consistent with EIN3 being a transcriptional activator, fusion of the full size protein with the GAL4 DNA-binding domain (BD) activate transcription of the *LacZ* reporter gene, indicated that EIN3 possesses activation domains that are functional in yeast. To avoid this activation of the reporter gene, an EIN3 derivative containing amino acids 53 to 257, fused to the GAL4-BD, was used as a "bait." As a "prey," the GAL4-activation domain was fused to an *Arabidopsis* cDNA library constructed using mRNA from etiolated seedlings (Kim *et al.*, 1997). Yeast strain Y190 transformed with the "bait" construct was subsequently transformed with the "prey" and four million independent transformants were screened for positive interactions, as described by Kim *et al.* (1997).

Twenty-six independent positive clones were obtained. The plasmids were recovered and their cDNAs were sequenced. Among them, six different clones corresponded to *EIN3*. All positives were re-tested by directly transforming yeast with both the original "bait" and the recovered "prey." Figure 6B shows an example of the interaction with one of these positives that included amino acids 113 to 628 of EIN3. Since the "bait" contains EIN3 residues 53 to 257, the dimerization domain was localized as residing between amino acids 113 and 257. These results also confirmed that

WO 00/32761

PCT/US99/28104

interaction of the EIN3-binding domain with DNA is not required for protein dimerization.

To examine whether other members of the EIN3/EIL family are also able to bind DNA, EMSA experiments were performed using *in vitro* translated EIL1, EIL2 and EIL3 proteins, and a DNA fragment containing the EBS or a mutant version. Consistent with the ability of EIL1 and EIL2, but not EIL3, to complement the *ein3-1* mutation in transgenic plants, both EIL1 and EIL2, but not EIL3, were able to specifically recognize the EBS element (Figure 6C).

To assess whether EIN3 and the EILs were capable of forming heterodimers, DNA-binding experiments using all combinations of co-translated EIN3/EILs proteins were performed. While mobility shifted bands corresponding in position to homodimeric forms of EIN3, EIL1 and EIL2 were observed, DNA-protein complexes with intermediate mobility were not seen, indicating that these proteins do not form heterodimers.

Example 6: ERF1 Is a GCC-Box Binding Protein

The preceding examples demonstrated that EIN3 is a transcriptional activator that is both necessary and sufficient for *ERF1* expression. Therefore, it was predicted that ERF1 would play a role in directing the expression of target genes containing the GCC element. However, at least one EREBP, involved in the regulation of cold and drought response, is known to bind a DNA sequence unrelated to the GCC element (*i.e.*, C-box/DRE element; Stockinger *et al.*, *Proc. Natl. Acad. Sci. USA* 94:1035-1040 (1997)).

To determine whether ERF1 contained a functional DNA-binding domain, which would be able to interact with the GCC element in a sequence-specific manner, DNA-binding experiments were performed with *in vitro* translated ERF1 protein. Radiolabeled promoter fragments of the ethylene-regulated *Arabidopsis* basic-chitinase (Samac *et al.*, 1990) and bean chitinase5B genes (Broglie *et al.*, 1989), were incubated with ERF1 and analyzed by EMSA. To examine the specificity of the interaction, a mutated version of the promoter fragments were also used, in which the cytosines of the GCC box element were substituted by thymines.

WO 00/32761

PCT/US99/28104

As shown in Figure 7, ERF1 was able to specifically bind the promoter fragments containing the GCC element, whereas no binding was observed to the mutant sequences. The lower band in each lane containing ERF1 and the wild-type element apparently corresponds to a truncated form of ERF1 since two major bands were obtained as products of the *in vitro* translation of *ERF1* mRNA.

Example 7: Downstream Activation of Ethylene Responses by ERF

To further evaluate the role of ERF1 in the ethylene signaling pathway, transgenic plants were constructed which constitutively expressed *ERF1* mRNA under control of a CaMV 35S promoter. T2 segregants of these transgenic lines were examined for ethylene response phenotypes.

Out of a total of 26 independent lines, plants from 9 lines displayed phenotypes similar to those observed in the constitutive ethylene response mutant *ctr1*, or in EIN3- or EIL1-overexpressing plants. Etiolated *35S::ERF1* seedlings grown in hydrocarbon-free air showed inhibition of root and hypocotyl elongation, typical of the response to treatment with ethylene (Figure 8A-H). However, the apical hook did not display exaggerated curvature typical of an ethylene response. The cotyledons of ERF1-expressing seedlings were still appressed and many were still encapsulated in the seed coat.

Consistent with this phenotype, *HOOKLESS1*, an ethylene response gene required for apical hook curvature (Lehman *et al.*, *Cell* 85:183-194 (1996)), was not expressed in ERF1-overexpressing plants. Expression of only a partial seedling triple response phenotype in these lines is consistent with a role for ERF1 in mediating a subset of the ethylene responses. *ERF1* may act along with other genes, *e.g.*, *EREBPs* and others, to fully mediate the various seedling responses to ethylene.

As adults, *35S::ERF1* transgenic plants showed an extreme dwarf phenotype similar to the constitutive ethylene response mutant *ctr1* and to EIN3/EIL1-overexpressing transgenic plants (Figure 9A-G). As in the case of the quadruple ethylene receptor knockout mutant (Hua and Meyerowitz, *Cell*, 1998), plants from several *ERF1*-expressing lines showed extreme inhibition of cell enlargement, and ultimately the plants wilted and died before bolting.

To determine whether the "ethylene" morphology displayed by these plants was the consequence of ethylene overproduction, or due to constitutive activation of the signaling pathway, the 35S::*ERF1* gene was also introduced into several mutant backgrounds (*ein2-5*, *ein2-17*, *ein2-26*, *ein3-1*, *ein3-3* and *ein5-1*) that suppress phenotypes resulting from ethylene overproduction. In all cases, the transgenic plants displayed a morphology indistinguishable from that of 35S::*ERF1*-expressing wild-type plants (Figures 9 and 10). Thus, the observed morphology evoked by expression of *ERF1* was not a consequence of ethylene production; rather, like CaMV 35S::*EIN3* expression, the morphology resulted from constitutive activation of the response pathway. Moreover, because there was an absence of the requirement for functional EIN2, EIN3 or EIN5 proteins for the constitutive activation phenotype, the morphology results provided strong evidence for the downstream location of ERF1.

To confirm whether the observed morphology in the 35S::*ERF1* lines was due to activation of ethylene response, the expression of several ethylene-regulated genes was examined. As expected, the ethylene induced accumulation of mRNAs for two ethylene response genes, basic-chitinase and *PDF1.2* was completely blocked in a strong mutant (*ein2*), or was significantly reduced in the weak ethylene insensitive mutants (*ein3* or *ein5*) (Figure 10A). In each of five independently derived transgenic *ERF1*-overexpressing 5 week-old, air grown plants, high level constitutive expression of mRNAs for basic-chitinase and *PDF1.2* was observed (Figure 10A). Total RNA (5 µg) was loaded per lane in the middle and right panels, and 50 µg in the left panel of Figure 10A. Moreover, constitutive expression of chitinase and *PDF1.2* mRNA was observed when the 35S::*ERF1* gene was introduced into three different ethylene insensitive mutant backgrounds. The *ein2*; 35S::*ERF1*, *ein3*; 35S::*ERF1* and *ein5*; 35S::*ERF1* transgenic lines all showed high level expression of mRNAs for these genes. In the case of *ein5-1* the lower expression of *ERF1* in one of the two transgenic lines correlated with the lower expression of *PDF1.2* and basic-chitinase, and with the weaker constitutive ethylene response phenotype.

The effect of 35S::*ERF1* expression on a chitinase promoter gene fusion, *CH5B*::*GUS*, was also examined. This well-characterized ethylene-responsive reporter gene has proven to be a reliable marker for ethylene-evoked transcription in bean

WO 00/32761

PCT/US99/28104

(Broglie *et al.*, 1989), and *Arabidopsis* (Chen *et al.*, *Plant Physiol.* 108:597-607 (1995)).

Three-week-old F1 plants derived from crosses between plants carrying the *CH5B::GUS* reporter gene and *ERF1*-overexpressing lines or wild-type plants were grown on agar plates and stained for GUS activity..

5 As revealed by intense staining in seedlings, high level GUS activity was observed in the presence of *35S::ERF1*, whereas no staining was detected in the control plants (absence of *35S::ERF1*) (Figure 10B). Moreover, introduction of the *35S::ERF1* construct into an ethylene-repressible enhancer-trap reporter line also resulted in inhibition of GUS expression in the absence of ethylene, confirming repressed
10 transcription of the ethylene-regulated gene by ERF1 expression.

These results confirm that ERF1 expression is sufficient to promote (or repress) transcription of ethylene-regulated target genes in a variety of plant tissues.

In sum, rapid EIN3-dependent induction of *ERF1* expression in response to ethylene, binding of EIN3 to the ERF1 promoter, and constitutive expression of *ERF1* in
15 *EIN3*-overexpressing plants support the conclusion that *ERF1* is an immediate target of EIN3. Binding of ERF1 to the GCC element in the promoters of ethylene-regulated genes, and constitutive activation of ethylene response genes and phenotypes in both etiolated seedling and adult plants in the ERF1 gain-of-function experiments further define ERF1 as a downstream ethylene signaling pathway gene. The sequential action of
20 EIN3 (or EILs) and ERF1 DNA binding proteins adds a new level of complexity in the regulatory hierarchy of the ethylene-signaling pathway. Thus, the existence of this hierarchy of enzymes in the signaling pathway for ethylene provides a means to finely regulate the complex plant response to this gaseous plant growth regulator/stress signal.

— The disclosures of each patent, patent application and publication cited or
25 described in this document are hereby incorporated herein by reference, in their entirety.

Various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

WO 00/32761

PCT/US99/28104

What is claimed is:

1. An isolated nucleic acid encoding a plant ethylene response factor, which is activated by EIN3 or an EIN3-like (EIL) peptide in the plant ethylene signaling pathway, wherein the activated factor binds to a target GCC-box in a secondary ethylene response gene.
2. The nucleic acid according to claim 1, comprising *ERF1*.
3. The nucleic acid according to claim 2, further comprising mutants, derivatives, homologues or fragments of *ERF1*, encoding an expression product having ERF1-activity.
4. The nucleic acid according to claim 2, comprising SEQ ID NO:1.
5. A purified preparation of a polypeptide encoded by the nucleic acid of any of claims 2-4.
6. The polypeptide according to claim 5, comprising ERF1 and homologues, analogs, derivatives or fragments thereof, having GCC-box binding activity in a target secondary response gene.
7. The polypeptide according to claim 6, comprising SEQ ID NO:2.
8. The polypeptide according to claim 7, wherein ERF1 GCC-box binding activity is *EIN3*- or *EIL*-dependent.
9. A recombinant cell comprising the isolated nucleic acid of any of claims 1-4.
10. A vector comprising the isolated nucleic acid of any of claims 1-4.

11. An antibody specific for a plant ERF1 polypeptide, and homologues, analogs, derivatives or fragments thereof, having GCC-box binding activity in a target secondary response gene.
12. An isolated nucleic acid sequence comprising a sequence complementary to all or part of the nucleic acid sequence of one of claims 1-4, and to mutants, derivatives, homologues or fragments thereof encoding a GCC-box binding expression product in a target secondary response gene.
13. The nucleic acid according to claim 12 having antisense activity at a level sufficient to regulate, control, or modulate the secondary ethylene response activity of a plant, plant cell, organ, flower or tissue comprising same.
14. A plant, plant cell, organ, flower, tissue, seed, or progeny comprising nucleic acid according to any of claims 1-4 or 12-13.
15. A transgenic plant, the cells, organs, flowers, tissues, seeds or progeny of which comprise the nucleic acid according to any of claims 1-4 or 12-13.
16. A transgenic plant, the cells, organs, flowers, tissues, seeds or progeny of which comprise the recombinant nucleic acid according to claim 9.
17. A transgenic plant, the cells, organs, flowers, tissues, seeds or progeny of which comprise the polypeptide according to any of claims 5-8.
18. An isolated nucleic acid of one of claims 1-4 or 12-13, further comprising a plant ERF1 promoter sequence, or a fragment thereof having ERF1 promoter activity.

WO 00/32761

PCT/US99/28104

19. A vector comprising the isolated nucleic acid of any of claims 1-4 or 12-13 or 18.
20. The isolated nucleic acid of claim 18, further comprising a reporter gene operably
21. fused thereto, or a fragment thereof having reporter activity.
22. A transgenic plant, the cells, organs, flowers, tissues, seed, or progeny of which comprise a transgene comprising an isolated nucleic acid comprising an ERF1 promoter sequence.
23. A method for manipulating in a plant the nucleic acid according to any of claims 1-4 or 12-13 to permit the regulation, control or modulation of the ethylene response in said plant.
24. The method according to claim 22 wherein said regulation, control or modulation initiates or enhances the germination, cell elongation, sex determination, flower or leaf senescence, flower maturation, fruit ripening, insect, herbicide or pathogen resistance, abscission, or response to stress, injury or pathogens in said plant.
25. The method according to claim 22, wherein said regulation, control or modulation inhibits or prevents the germination, cell elongation, sex determination, flower or leaf senescence, flower maturation, fruit ripening, insect, herbicide or pathogen resistance, abscission, or response to stress, injury or pathogens in said plant.

26. A method of identifying a compound capable of affecting the ethylene response in the ethylene signaling system in a plant comprising:
- providing a cell comprising an isolated nucleic acid encoding a plant *ERF1* sequence, having a reporter sequence operably linked thereto;
 - adding to the cell a compound being tested; and
 - measuring the level of reporter gene activity in the cell, wherein a higher or lower level of reporter gene activity in the cell compared with the level of reporter gene activity in a second cell to which the compound being tested was not added is an indicator that the compound being tested is capable of affecting the expression of a plant *ERF1* gene.
27. A method for generating a modified plant with enhanced ethylene response activity as compared to that of comparable wild type plant comprising introducing into the cells of the modified plant an isolated nucleic acid encoding *ERF1*, wherein said *ERF1* nucleic acid binds to the GCC-box, thereby activating target secondary ethylene response genes of the modified plant.
28. A method for generating a modified plant with enhanced ethylene response activity as compared to that of comparable wild type plant, comprising introducing into *EIN3*- or *EIL*-defective or deficient cells of the modified plant an isolated nucleic acid encoding *EIN3* or *EIL*, wherein said *EIN3* or *EIL* nucleic acid activates the *ERF1* gene, thereby permitting activation of target secondary ethylene response genes of the modified plant.

29. A method for generating a plant with diminished or inhibited ethylene response activity as compared to that of a comparable wild type plant comprising binding or inhibiting the ERF1 molecules within the cells of a modified plant by introducing into said cells an isolated nucleic acid encoding a complementary nucleic acid to all or a portion of *erf1*, wherein said *erf1* nucleic acid would otherwise bind to the GCC-box, thereby activating target secondary ethylene response genes of the modified plant.
30. A method for generating a plant with diminished or inhibited ethylene response activity as compared to that of a comparable wild type plant comprising binding or inhibiting the ERF1 molecules within the cells of a modified plant by introducing into said cells an antibody to all or a portion of ERF1, wherein said ERF1 polypeptide would otherwise bind to the GCC-box, thereby activating target secondary ethylene response genes of the modified plant.
31. A method for generating a plant with diminished or inhibited ethylene response activity as compared to that of a comparable wild type plant comprising binding or inhibiting the EIN3 or EIL molecules within the cells of a modified plant by introducing into said cells an antibody to all or a portion of EIN3 or EIL, wherein said EIN3 or EIL polypeptide would otherwise activate the expression of ERF1, thereby permitting activation of target secondary ethylene response genes of the modified plant.

WO 00/32761

PCT/US99/28104

32. A method for manipulating the expression of ERF1 in a plant cell comprising:
operably fusing the nucleic acid *ERF1* or an operable portion thereof to a plant promoter sequence in the plant cell to form a chimeric DNA, and
generating a transgenic plant, the cells of which comprise said chimeric DNA, where upon controlled activation of the plant promoter, manipulates expression of ERF1.

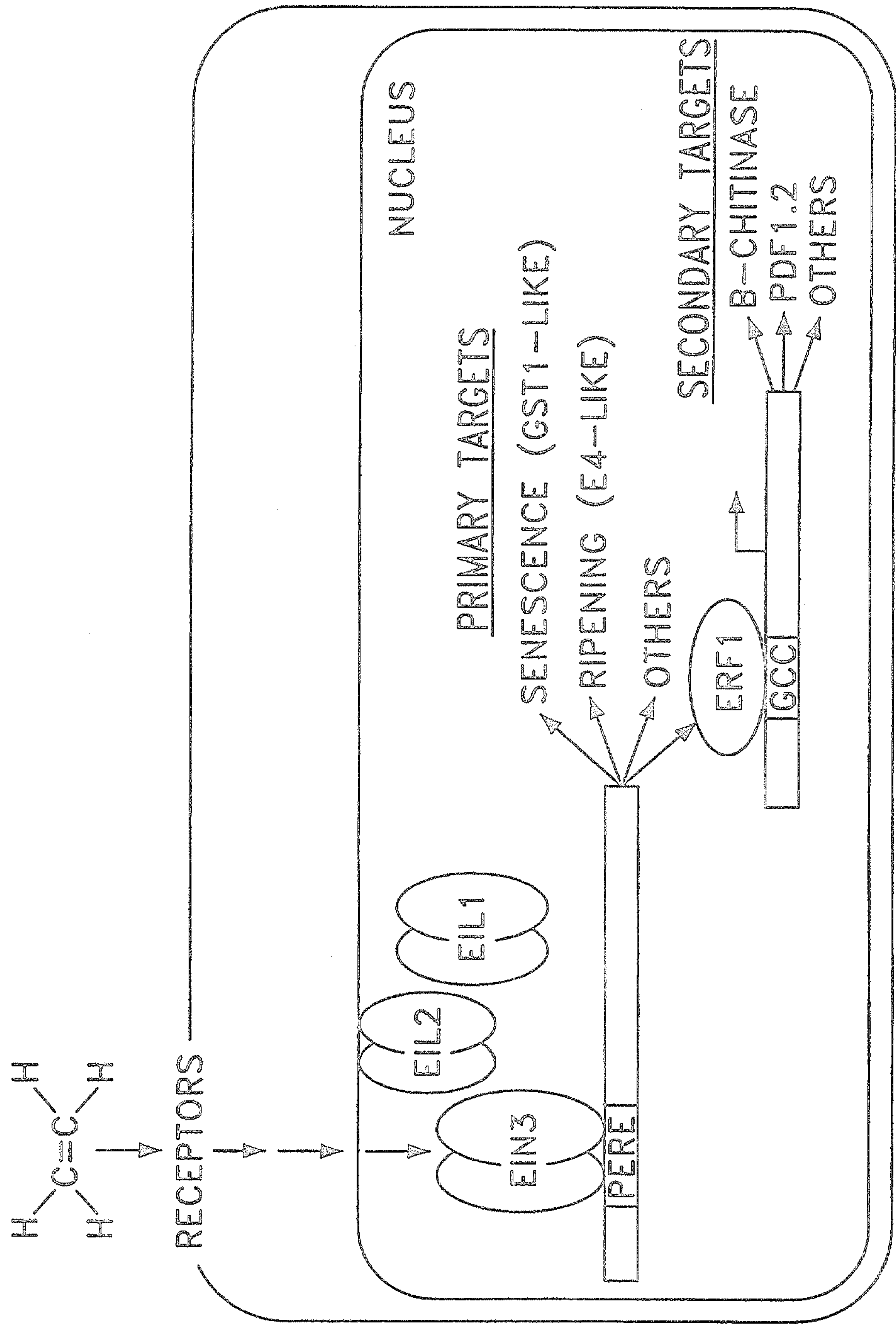


FIG. 1

2/10

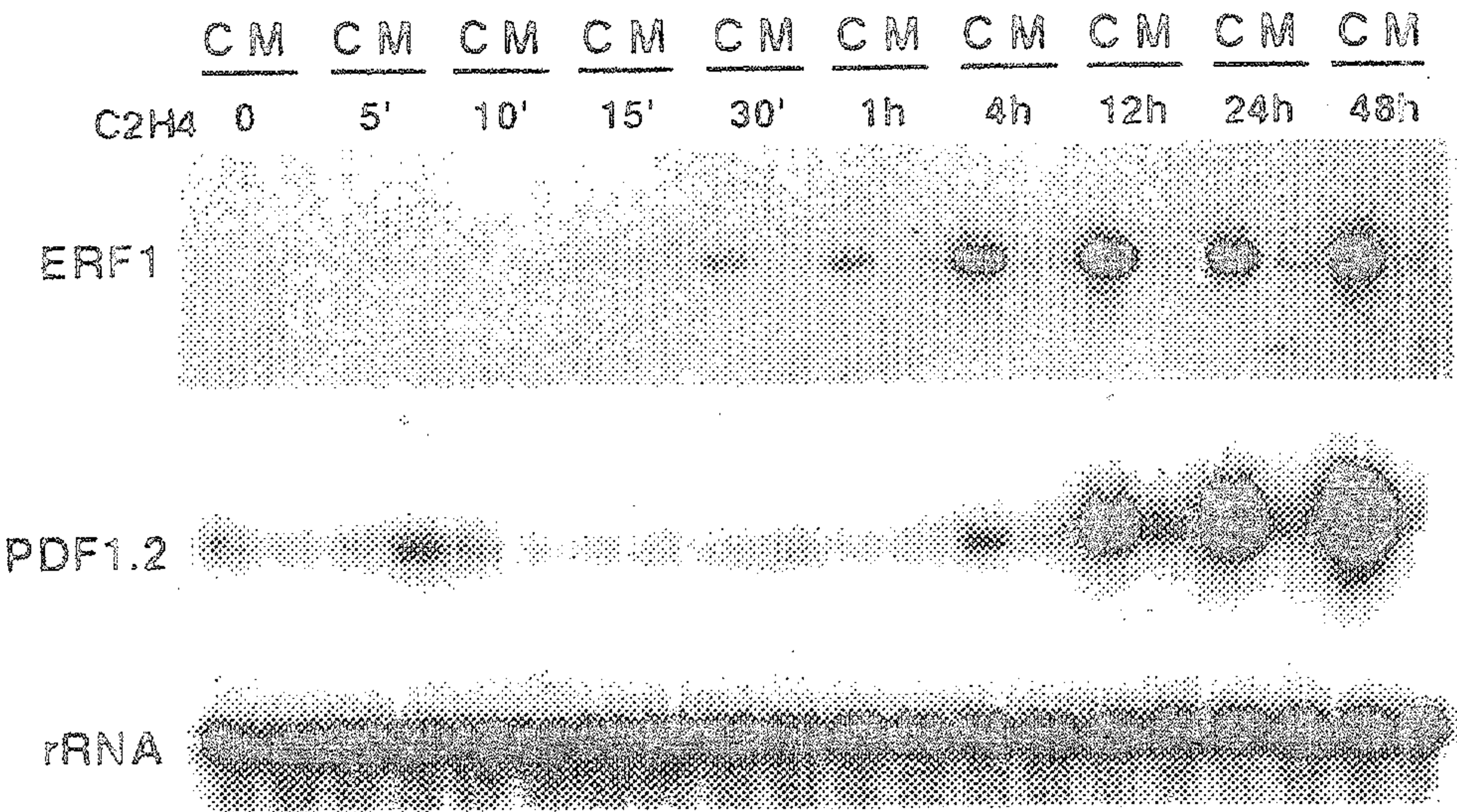


FIG. 2A

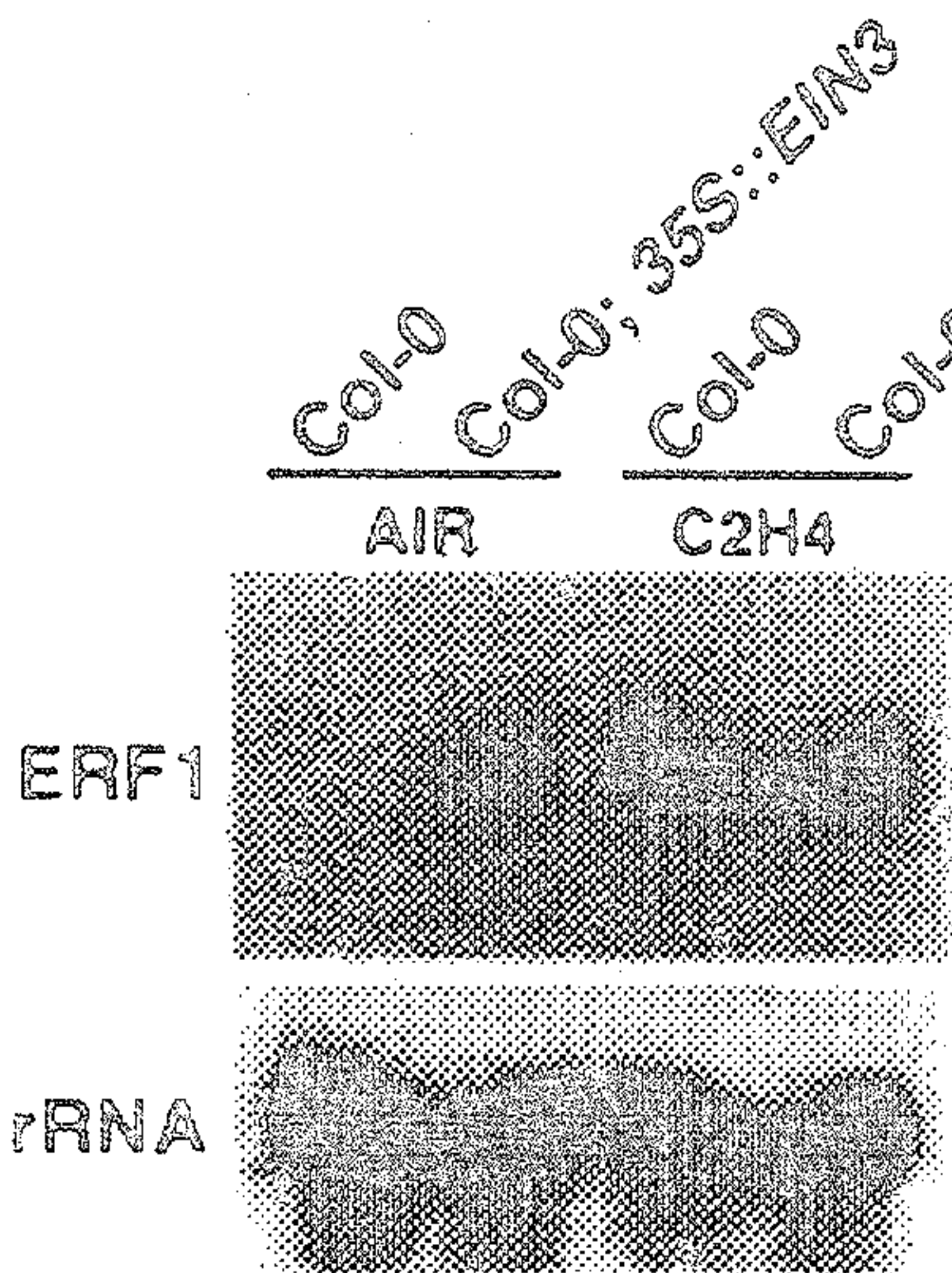
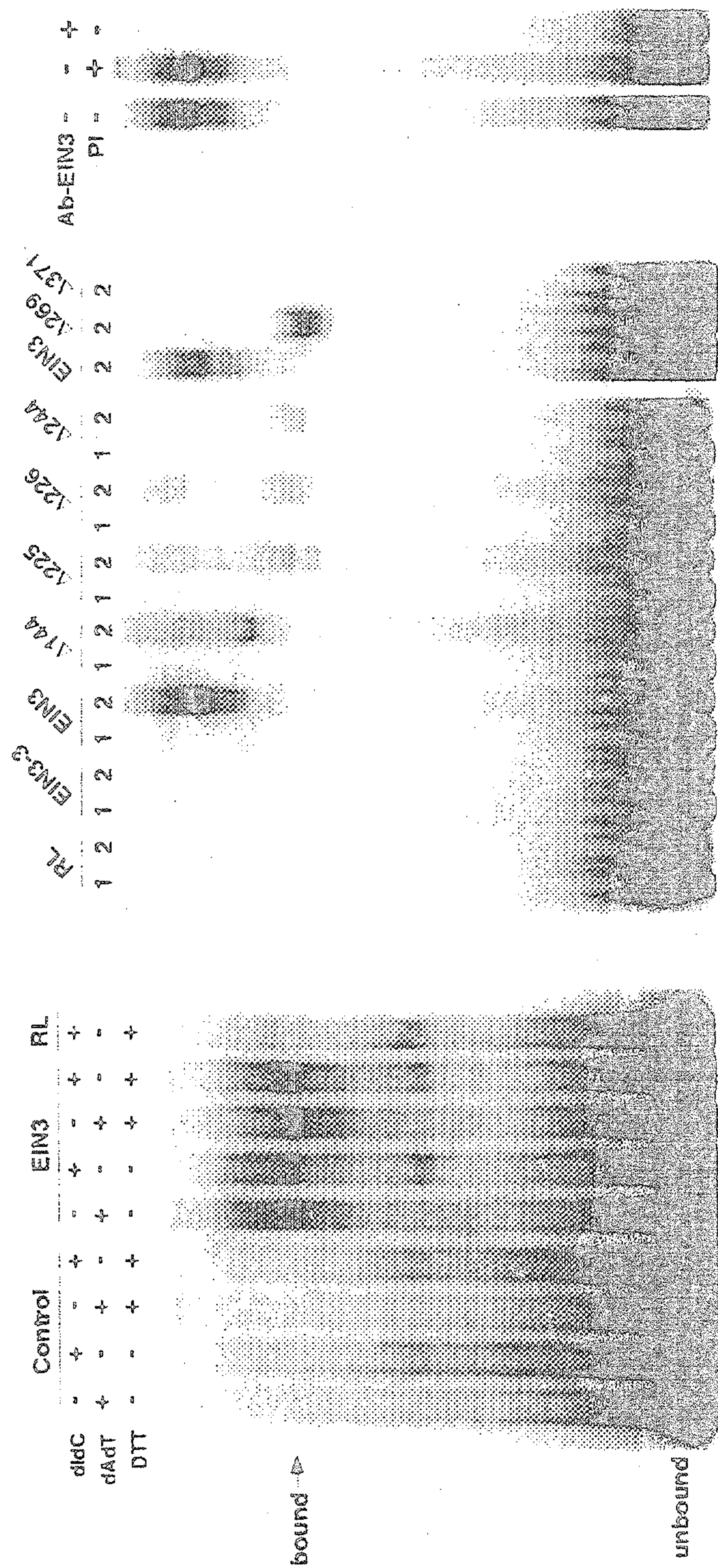


FIG. 2B



FIG. 2C

3/10



3A
FIG.

B
M
G
H
L



4/10

GTIGTTTGGGATTCAAGGGGCGATGTATCTTGAATCC
CAAC
AAAC
CCT
AGG
CCT
AAG
CTT
AAG
CTT
T
T
C
C
C
C
GTA
C
A
T
A
GAA

FIG. 4A

GST1⁻⁴⁹⁷ TATTTCAAAGAGCATGTAGCATCTCAAA⁻⁴⁶⁷
ERF1 GGATTCAAGGGGCGATGTATCTTGAATCC
E4⁻¹¹⁸ AAATTCAAATGGCATGTATAACGCATCT⁻⁸⁹

FIG. 4B

5/10

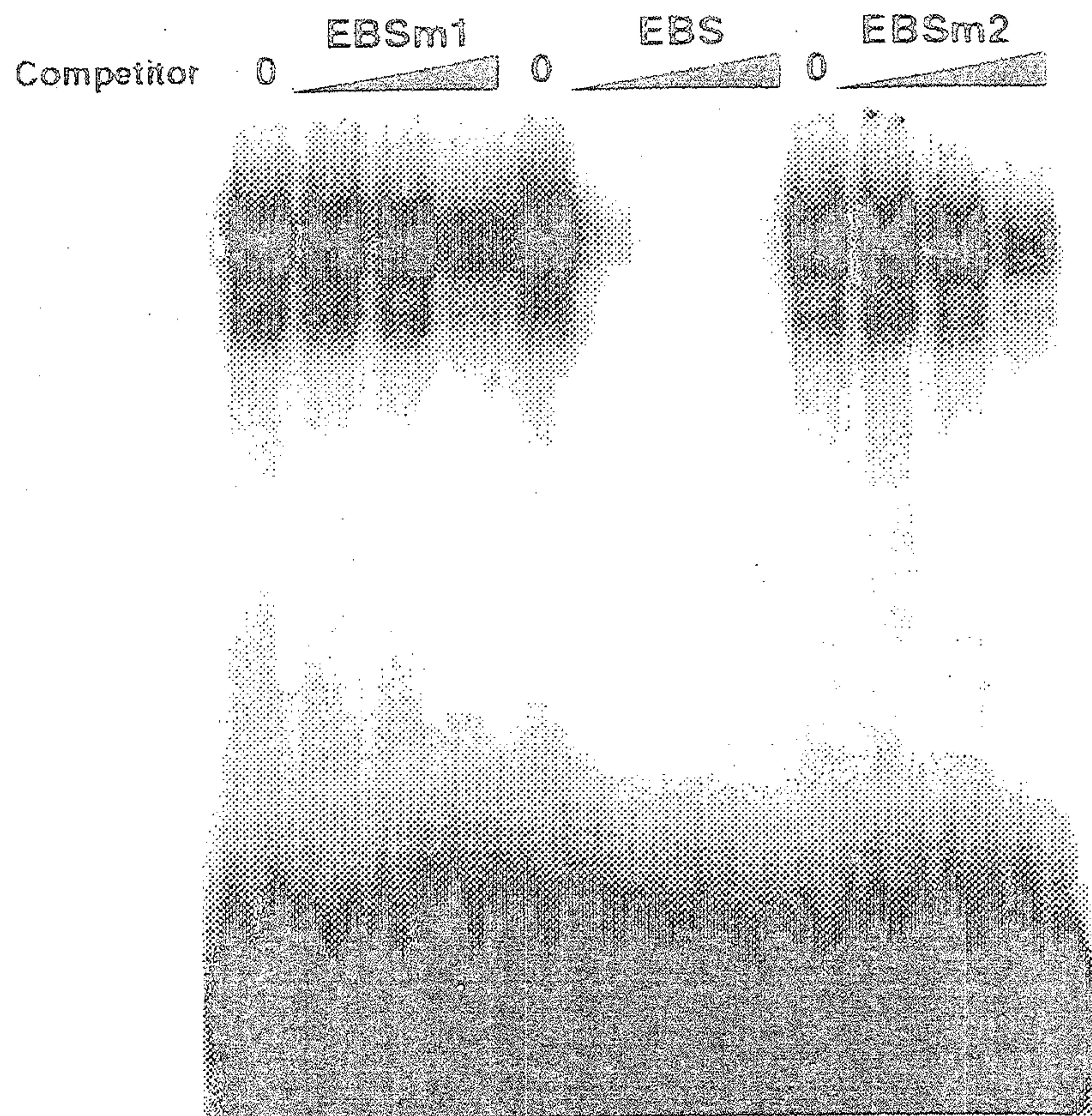


FIG. 5A

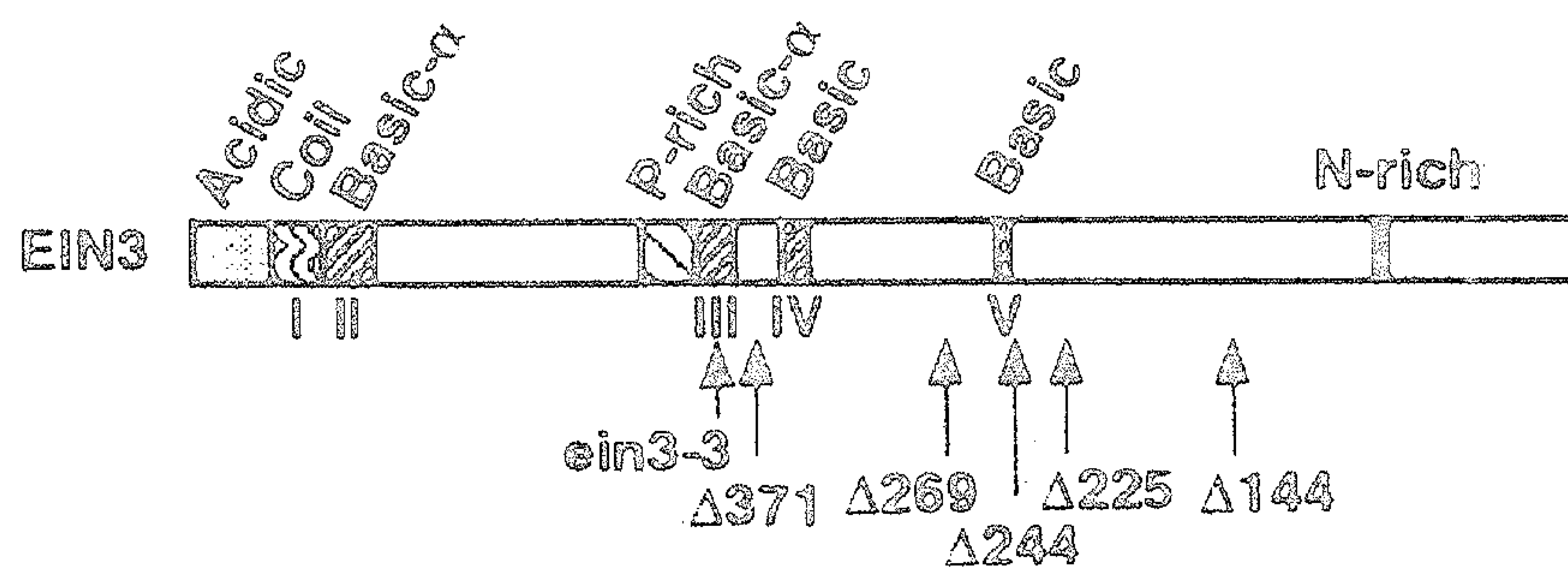


FIG. 5B

6/10

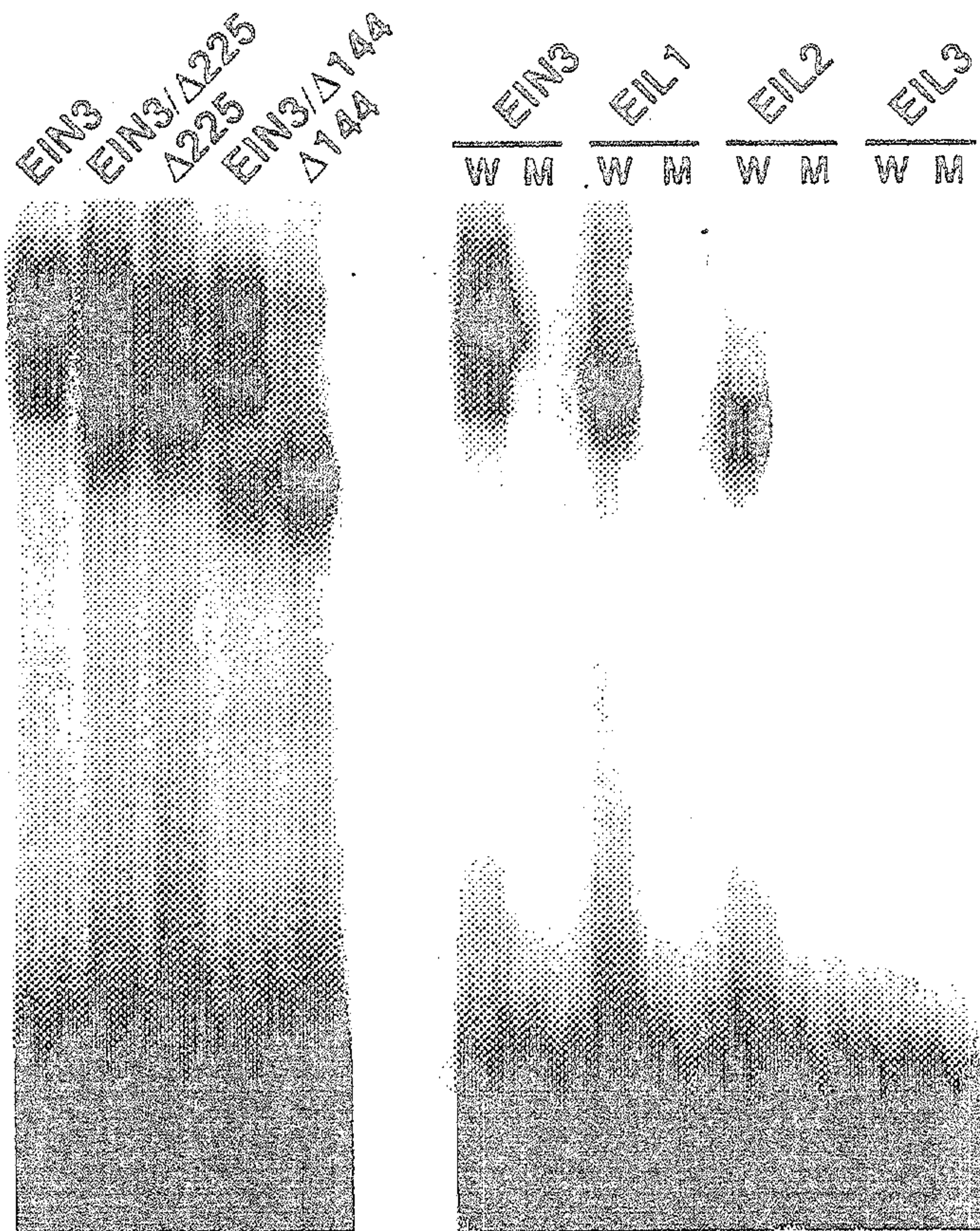


FIG. 6A

FIG. 6C

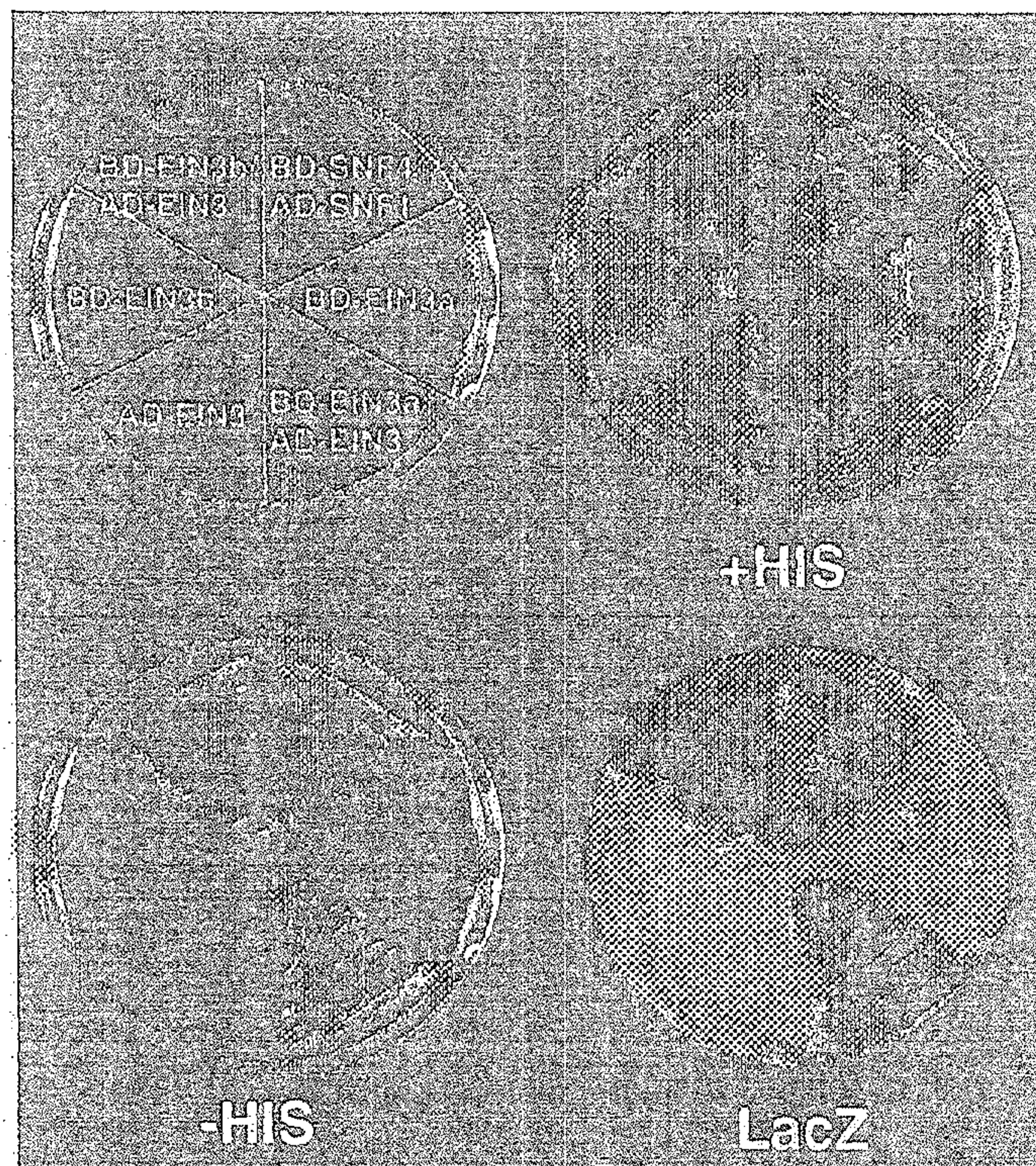


FIG. 6B

7/10

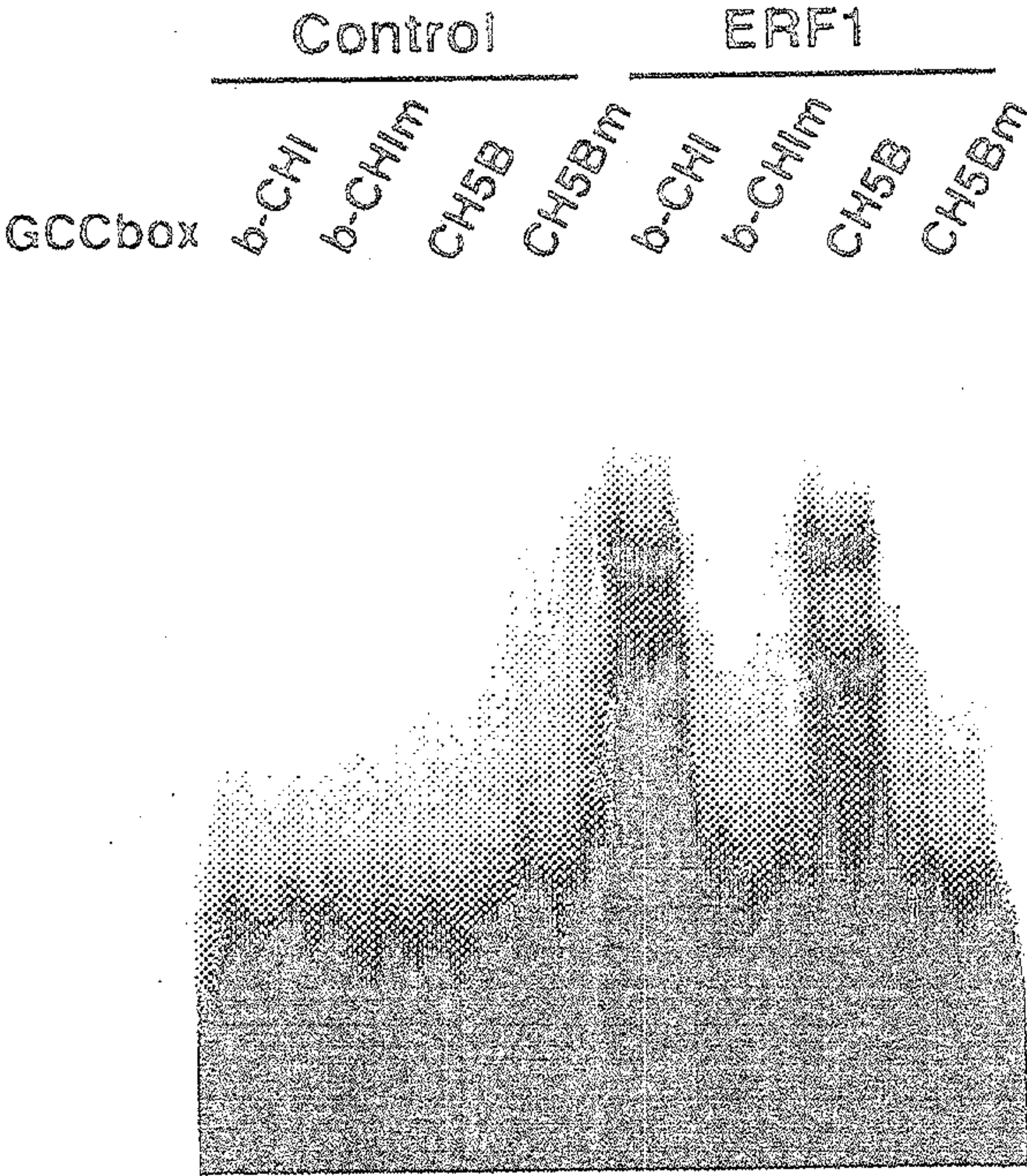


FIG. 7

8/10

Col-0

Col-0; 35S::ERF1

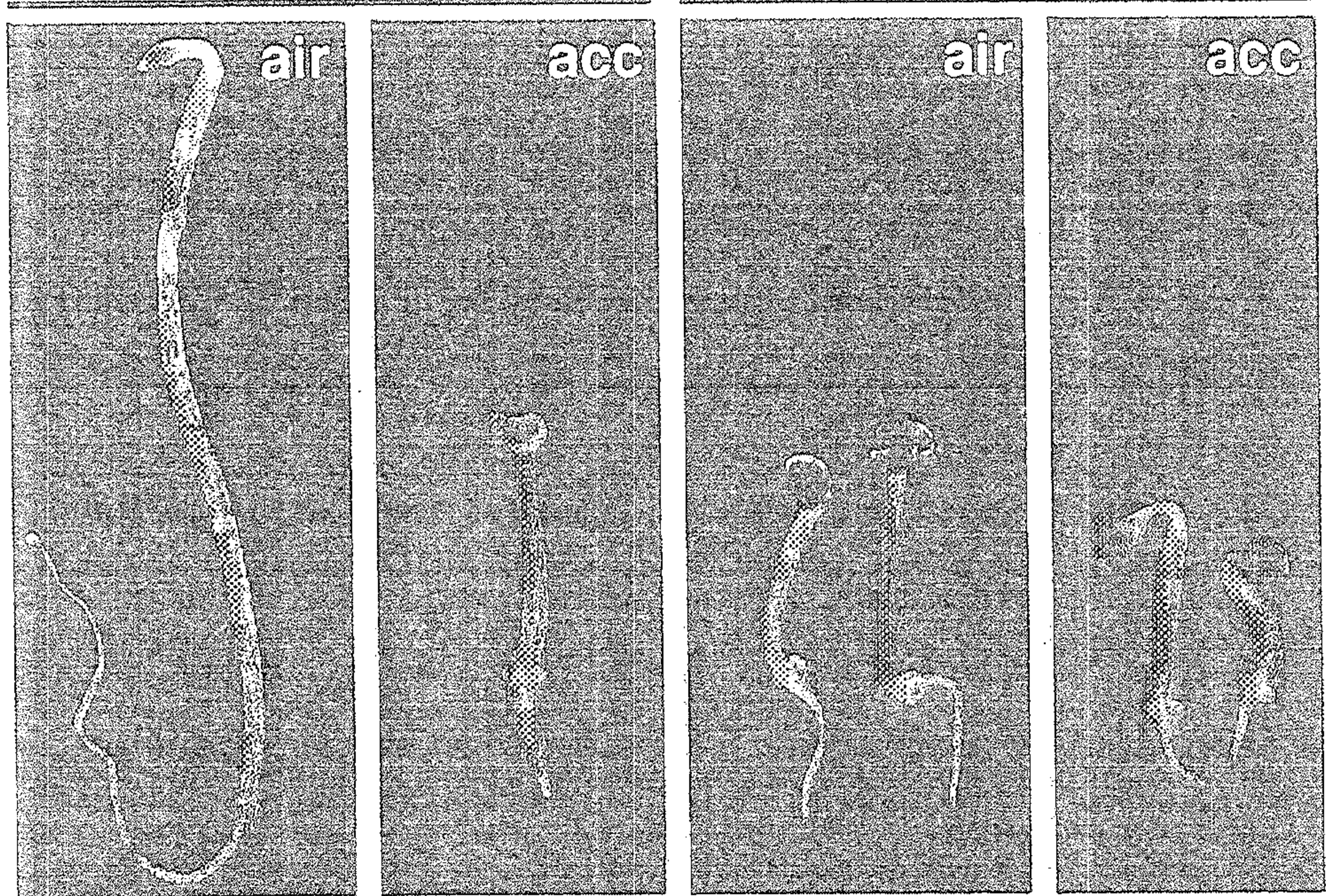


FIG. 8A FIG. 8B FIG. 8C FIG. 8D

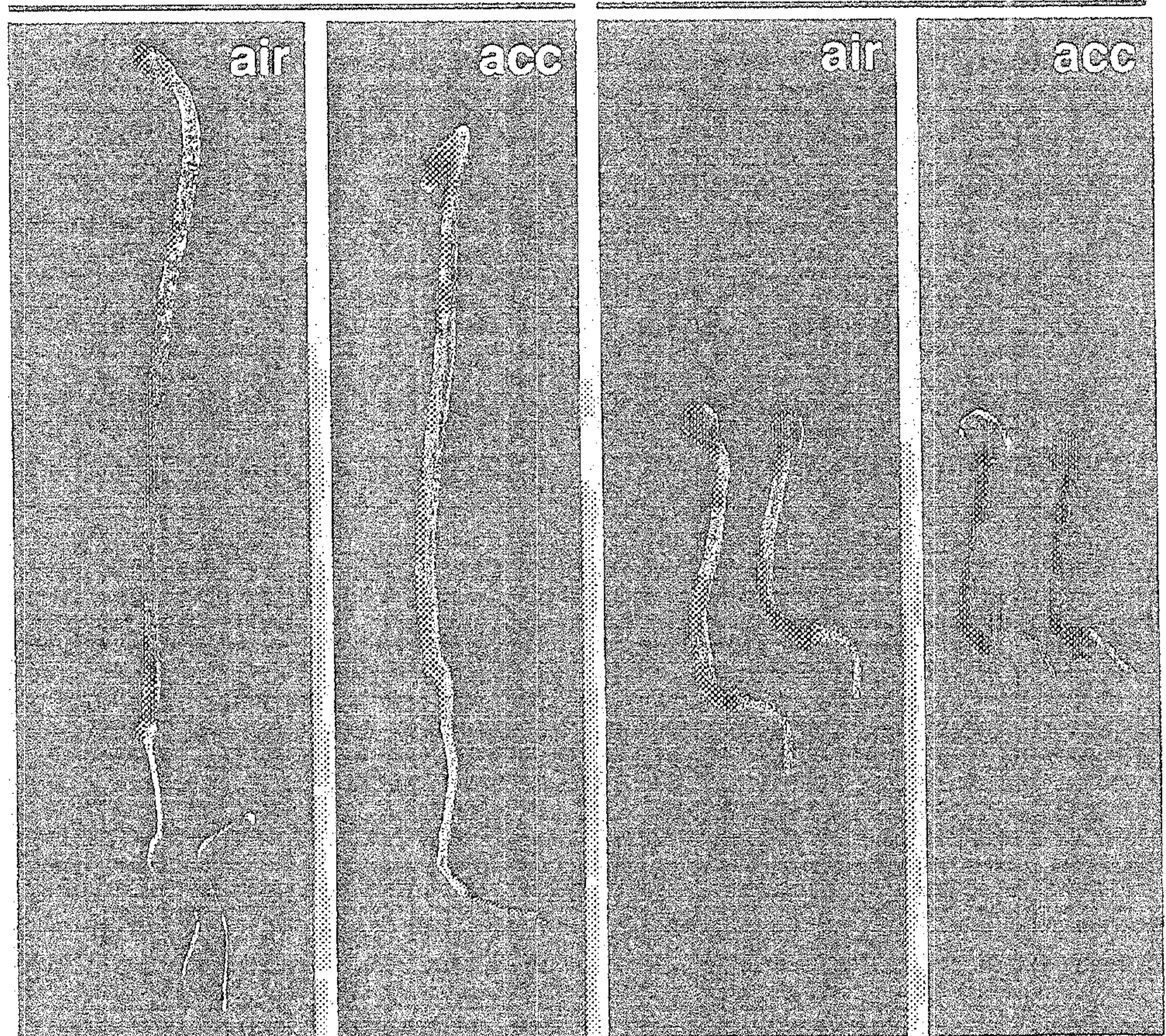
*ein3-1**ein3-1; 35S::ERF1*

FIG. 8E FIG. 8F FIG. 8G FIG. 8H

9/10

FIG. 9A

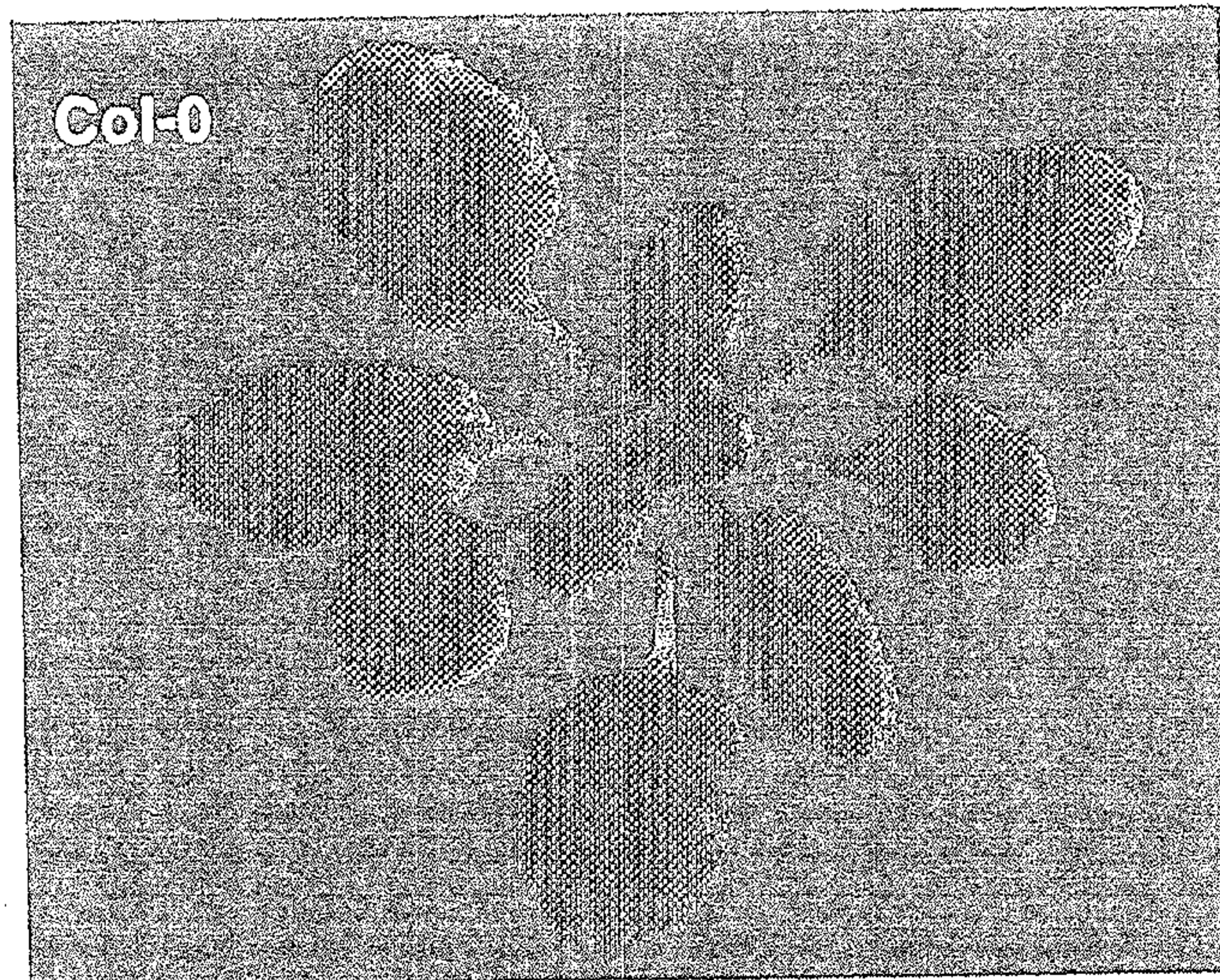


FIG. 9B

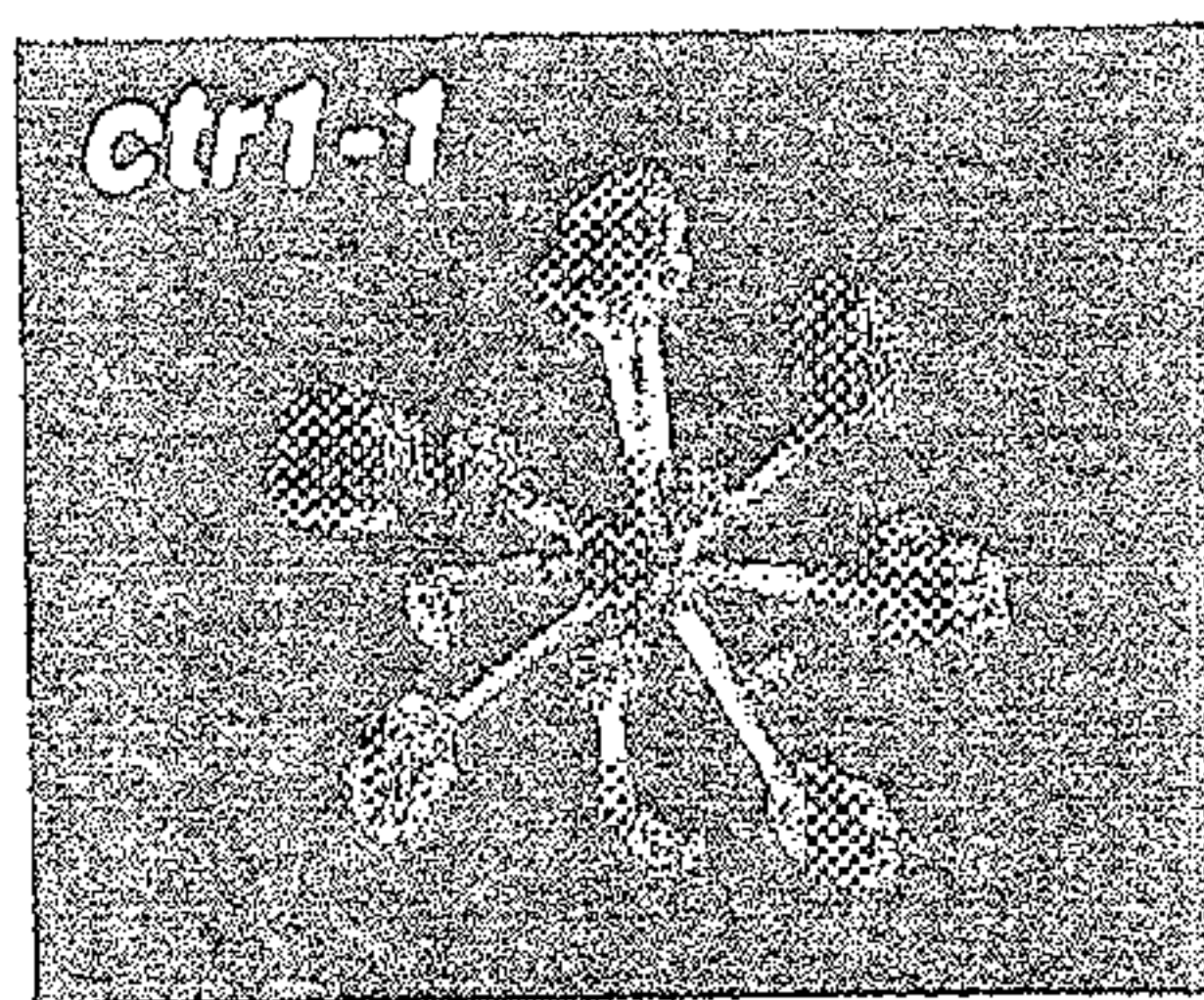


FIG. 9C

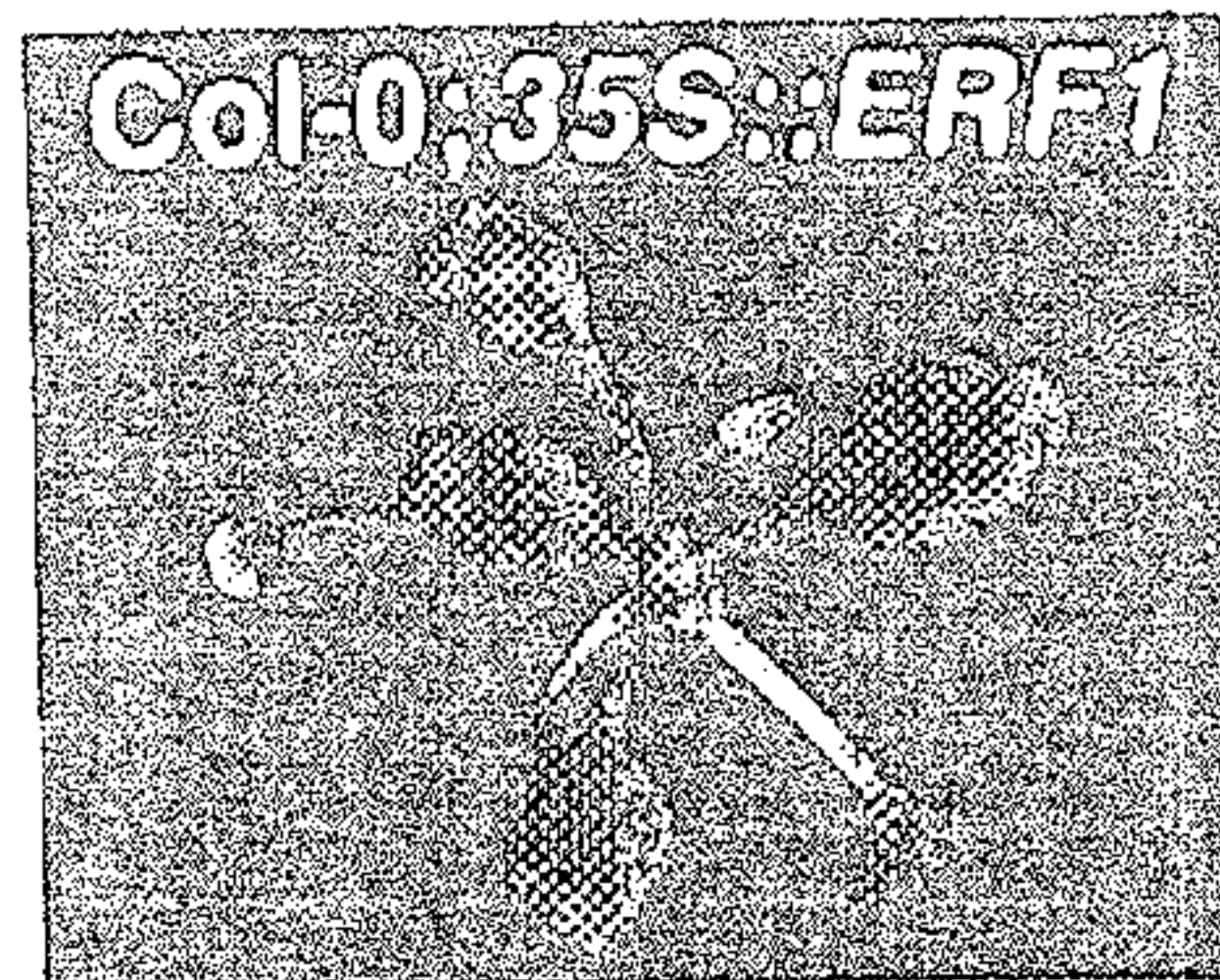


FIG. 9D

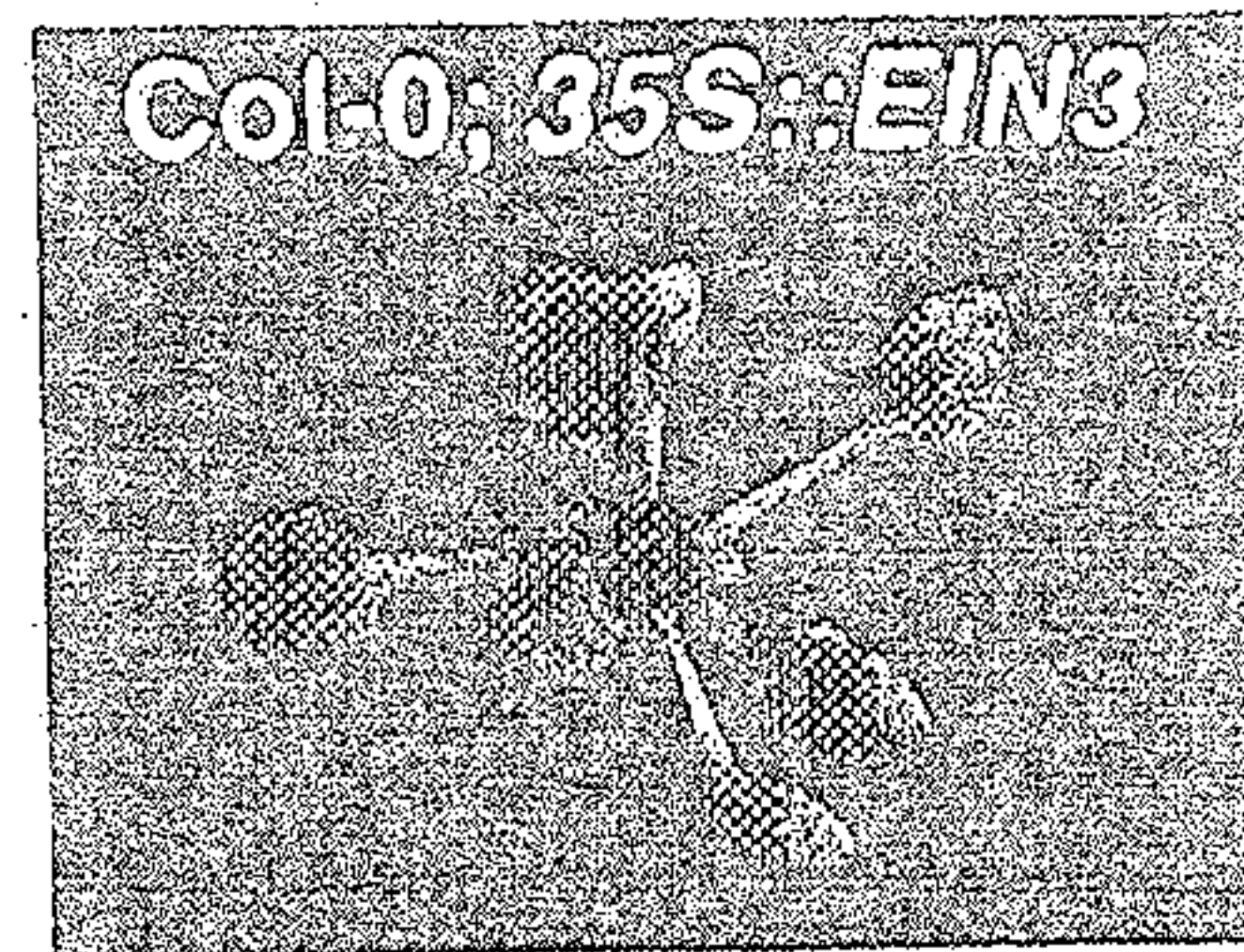
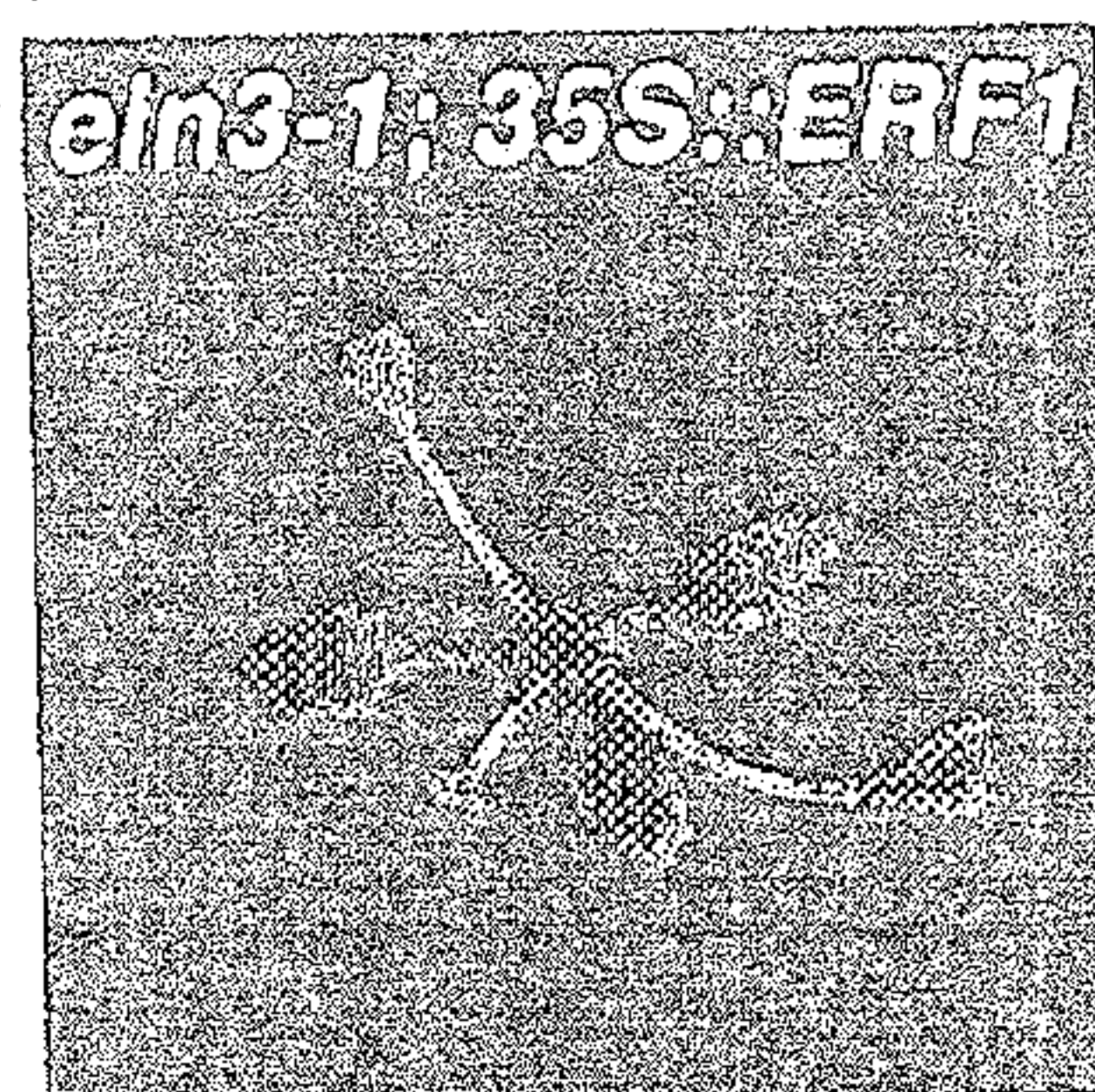


FIG. 9E



FIG. 9F

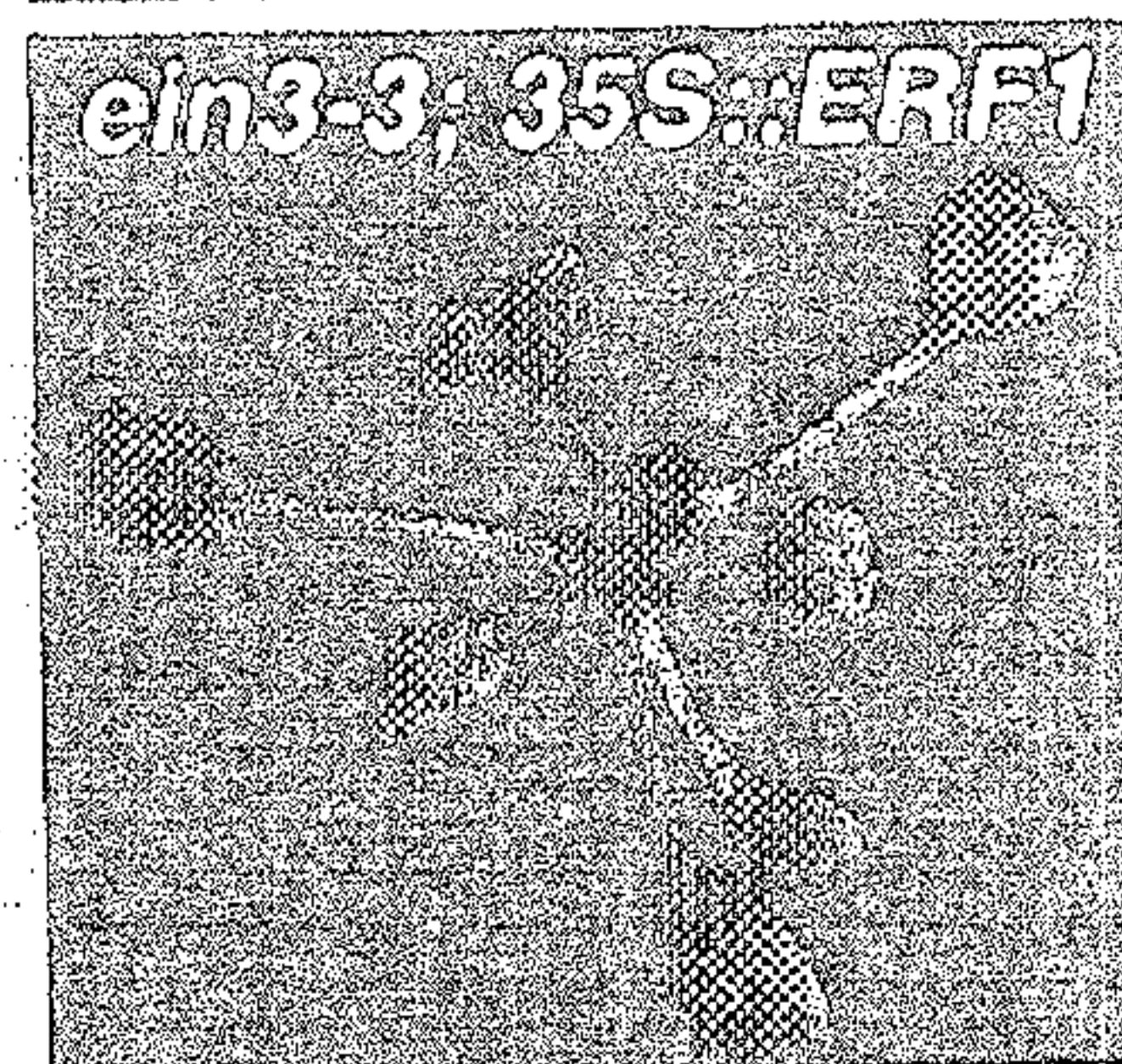


FIG. 9G

10/10

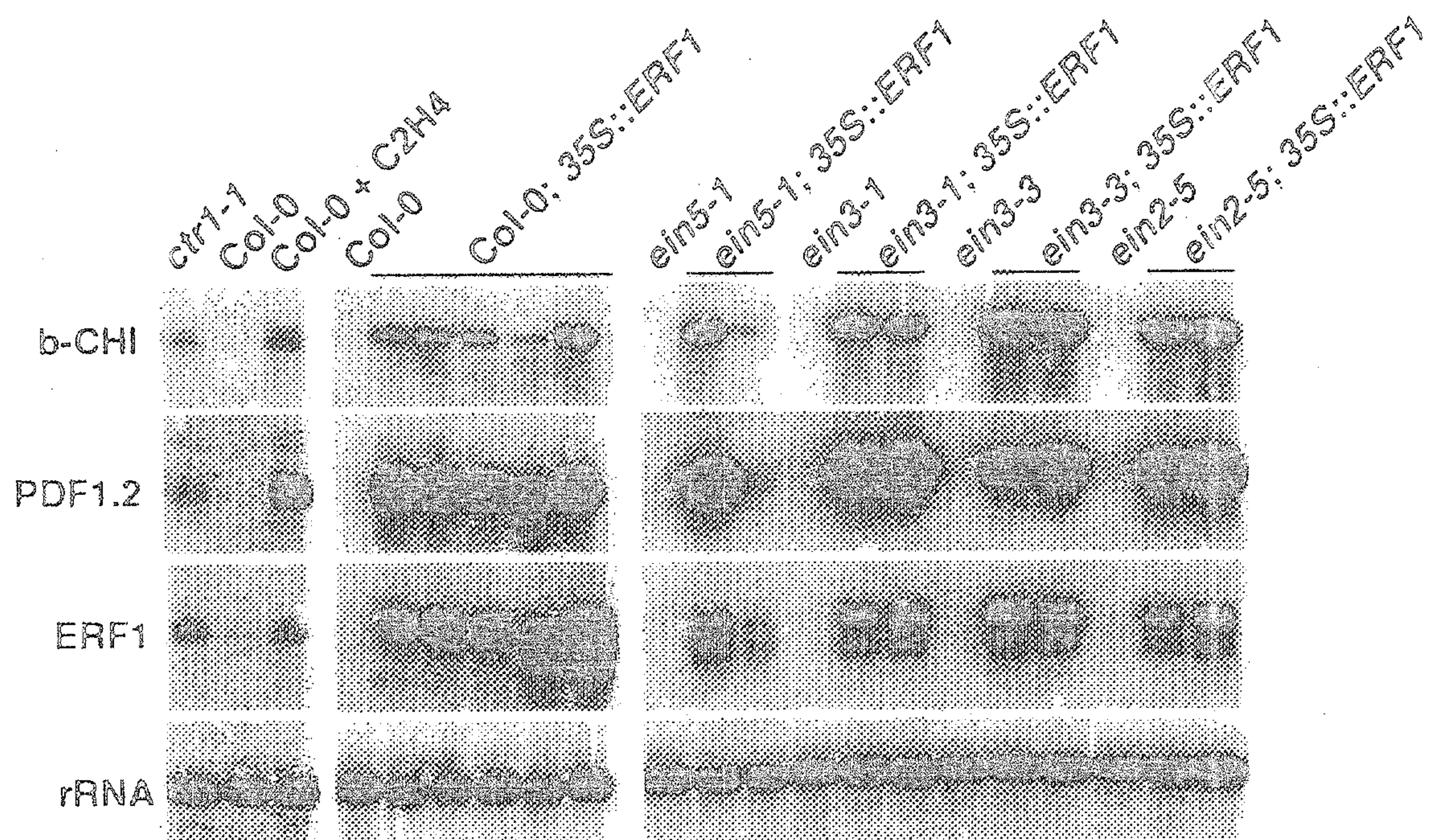


FIG. 10A



FIG. 10B