absorption at 292 nm was monitored. In some experiments, the LMW C18 fraction (containing 1/10 liver equivalent) was treated with 0.1 units of uricase at room temperature (26 °C) before being used for injection *in vivo*.

Immunization and CTL assays

Immunizations and CTL assays were done as described[10] except that the indicated adjuvants were used. Column fractions from 1–10% liver cytosol were injected without manipulation (GF250 column) or after drying and reconstitution (other columns). Pooled fractions without adjuvant activity were usually used as negative controls. For *in vitro* stimulation, splenocytes from immunized mice were stimulated with 10^{-8} M of RGPGRAFVTI or SIINFEKL peptide directly, and assayed as described[10].

In some experiments, mice were injected with uricase (10 μg per mouse) and allopurinol (800 μg per mouse) in the peritoneum daily for 3 d, and 3T3 cells were cultured in 50 μM allopurinol. The cells were irradiated with ultraviolet as described[10], frozen for 20 min, and varying numbers were then admixed with gp120–latex beads and injected subcutaneously (s.c.) together with allopurinol (20 μg) and uricase (0.4 μg) into the pretreated mice. Alternatively, mice treated with allopurinol plus uricase were immunized s.c. with varying numbers of bone-marrow-derived dendritic cells that had been pulsed with HIV gp120 peptide RGPGRAFVTI (1 μg ml^{-1} for 1 h). Controls were treated identically except that uricase and allopurinol were omitted from all steps.

Mass spectrometry

GC-MS and ESI-MS were done with a Quattro-II triple quadrupole (Waters) and a LCQ quadrupole ion trap (Finnigan) mass spectrometer, respectively. Technical parameters, as well the method of TMS derivatization, are given in the Supplementary Information.

Uric acid measurement

EL4 cells (10^8) were resuspended in 5 ml of culture media and incubated at 37 °C for 5 h after being treated with emetine[10] or cycloheximide for 1 h or heat-shocked at 45 °C for 20 min. 3T3 cells were either untreated or exposed to ultraviolet light for 5 min and incubated for 5 h. The cells were directly suspended in culture media (without washing) and disrupted by nitrogen cavitation followed by centrifugation. We determined the concentration of uric acid in cytosol, HPLC fractions and mouse plasma by either HPLC or a uric acid kit.

Dendritic cell culture and analysis

We cultured bone-marrow-derived dendritic cells as described[26]. After removing floating cells, cultures were stimulated as indicated for 6 or 24 h. Bound crystals were detached by incubation with either 0.5% heparin or 0.5 μM polyvinyl sulphate (100K) in PBS for 10 min. The cells were then collected by scraping and extensively washed. Staining and flow cytometry have been described[11]. MSU crystals were collected from parallel sets of wells without dendritic cells, washed with absolute alcohol and dissolved in 0.1 N NaOH. The amount of uric acid was determined by measuring ultraviolet absorption at 292 nM against a set of standard uric acid and NaOH solutions.

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Differential regulation of EIN3 stability by glucose and ethylene signalling in plants

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Glucose is a global regulator of growth and metabolism that is evolutionarily conserved from unicellular microorganisms to multicellular animals and plants[1]. In photosynthetic plants, glucose shows hormone-like activities and modulates many essential processes, including embryogenesis, germination, seedling development, vegetative growth, reproduction and senescence[2,3]. Genetic and phenotypic analyses of *Arabidopsis* mutants with glucose-insensitive (*gin*) and glucose-oversensitive (*glo*) phenotypes have identified an unexpected antagonistic interaction between glucose and the plant stress hormone ethylene. The ethylene-insensitive *etr1* and *ein2* mutants have *glo* phenotypes, whereas the constitutive ethylene signalling mutant *ctr1* is allelic to *gin4* (refs 4, 5). The precise molecular mechanisms underlying the complex signalling network that governs plant growth and development in response to nutrients and plant hormones are mostly unknown. Here we show that glucose enhances the degradation of ETHYLENE-INSENSITIVE3 (EIN3), a key transcriptional regulator in ethylene signalling[6,7], through the plant glucose sensor hexokinase[8]. Ethylene, by contrast, enhances the stability of EIN3. The *ein3* mutant has a *glo* phenotype, and overexpression of EIN3 in transgenic *Arabidopsis* decreases glucose sensitivity.

To test the hypothesis that glucose modulates ethylene signalling through the transcription factor EIN3, which acts downstream of ETR1 (ref. 9) and EIN2 (ref. 10), we tested the activity of EIN3 protein tagged with the Myc epitope in maize mesophyll proto-

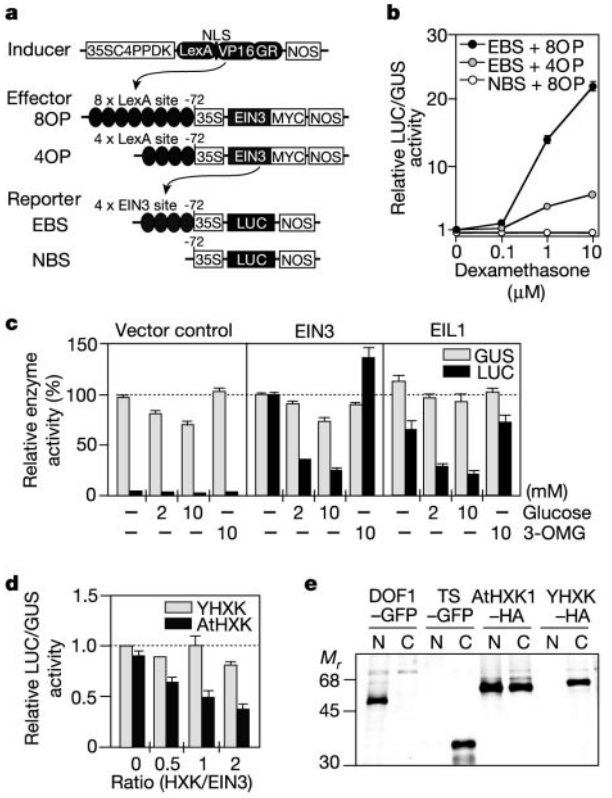

Figure 1 Functional analysis of EIN3 in maize protoplasts. **a**, The inducible expression system. 8OP and 4OP, LexA operator binding sites; LexA, LexA DNA-binding domain; NLS, nuclear localization signal; VP16, transcriptional activation domain; GR, glucocorticoid receptor. EBS indicates with, and NBS indicates without, the EIN3-binding site. **b**, Dosage-dependent transcriptional activation by EIN3. **c**, Glucose represses EIN3 and EIL1 activity. *EBS–LUC* (LUC) and *UBQ–GUS* (GUS) activities are shown. **d**, AtHXK1-dependent repression. **e**, Nuclear association of AtHXK1. Equal amounts of the nuclear (N) and cytosolic (C) fractions prepared from the same protoplast samples were analysed. TS, plastid transit peptide.

plasts, a well-established glucose-responsive plant-cell system[11]. To control EIN3 expression and to ensure specificity, we first used a glucocorticoid-receptor-inducible gene expression system based on the bacterial LexA repressor and its cognate DNA-binding site (ref. 12 and Fig. 1a). A luciferase (LUC) reporter gene controlled by four copies of the EIN3-binding sites (*EBS–LUC*)[7] and the 35S basal promoter[13] was used to quantify the activity of EIN3. In all transient expression assays, we used another reporter gene encoding β-glucuronidase (GUS) under the control of a constitutive ubiquitin promoter as an internal control[14,15]. We found that EIN3-dependent activation of *EBS–LUC* was markedly induced by dexamethasone in a concentration-dependent manner (Fig. 1b), showing that EIN3 is sufficient to activate transcription in maize protoplasts through a specific DNA-binding site that was previously defined *in vitro* for *Arabidopsis* EIN3 (ref. 7) and a tobacco EIN3 homologue TEIL[16].

To examine whether glucose influences EIN3 activity in maize protoplasts, we measured the transcriptional activity of EIN3 in the presence of glucose and a non-signalling glucose analogue, 3-*O*-methyl-glucose (3-OMG)[11], as an osmotic control. Expression of EIN3 from a constitutive CaMV 35S promoter strongly induced *EBS–LUC*, and only glucose at physiological concentrations (2 and 10 mM) significantly suppressed this *EBS–LUC* activity (Fig. 1c). Using the same cell system, we further tested the transcriptional activity and glucose sensitivity of an *Arabidopsis* EIN3-like protein (EIL1) that is known to rescue the *ein3* mutant phenotype[6]. EIL1 activated the expression of *EBS–LUC*, and this activation was repressed by glucose but not by 3-OMG (Fig. 1c). Thus, *Arabidopsis* EIN3 and EIL1 have similar DNA-binding and transcriptional activation functions and similar sensitivity to glucose.

We previously showed that *Arabidopsis thaliana* hexokinase (AtHXK1) acts as a glucose sensor with both metabolic and signalling functions[8]. Increasing the expression of AtHXK1 but not yeast HXK2 (YXHK2) led to stronger repression of EIN3-dependent *EBS–LUC* expression (Fig. 1d). Notably, immunoprecipitation analysis of the haemagglutinin (HA)-tagged HXKs showed that AtHXK1 but not YHXK2 could associate with the nuclear fractions of maize protoplasts (Fig. 1e). Fractionation was verified with two marker proteins tagged with green fluorescent protein (GFP), DOF1–GFP[14] and TP–GFP[17], which localize in the nucleus and chloroplasts, respectively. TP–GFP but not DOF1–GFP was released to the cytoplasmic fraction by 1% Triton X-100 (Fig. 1e).

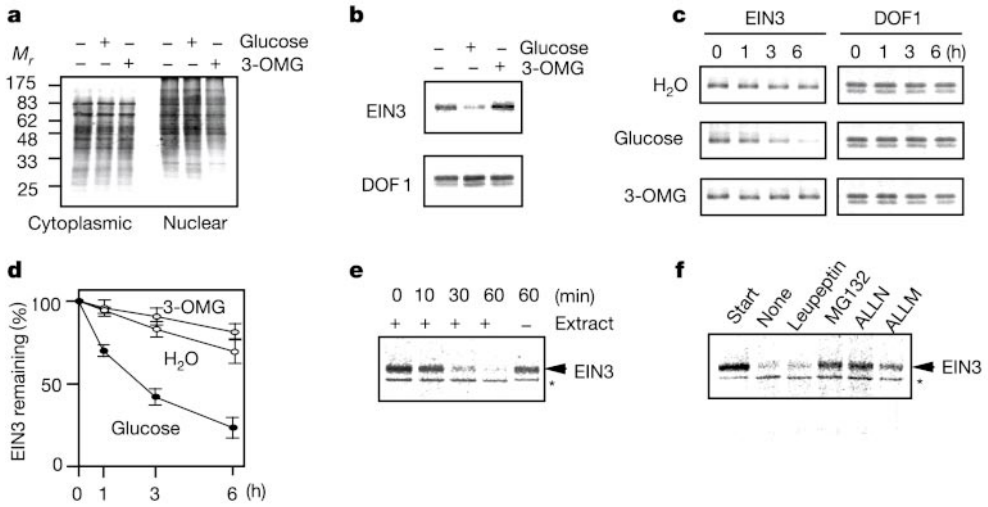

Figure 2 Glucose accelerates EIN3 degradation. **a**, Profiles of labelled proteins. Cytoplasmic fraction from 1.6×10^4 protoplasts or nuclear extract from 2×10^5 nuclei was loaded with a similar quantity of radioactivity. **b**, Accumulation of EIN3–Myc is affected by glucose. **c**, EIN3 degradation is enhanced by glucose *in vivo*. **d**, Plot of the amounts of EIN3 remaining. **e**, Time course of EIN3 degradation in a cell-free system. **f**, Cell-free degradation of EIN3 is inhibited by proteosome-specific inhibitors. Degradation reactions were allowed to proceed for 1 h with 10 μM of each inhibitor. Asterisks indicate nonspecific bands.

Although HXK is generally considered to be a soluble enzyme that is involved in glycolysis in the cytoplasm[8], AtHXK1 associated with the nucleus might directly or indirectly mediate glucose signalling into or out of the nucleus and might regulate EIN3 activity.

As *EIN3* gene expression is not induced by ethylene, it has been suggested that ethylene might activate EIN3 (ref. 6) or might regulate EIN3 protein expression[18]. To elucidate the molecular mechanism underlying the glucose-mediated repression of EIN3 activity, we investigated the stability of EIN3 protein. Constructs expressing EIN3 tagged with Myc and the unrelated maize transcription factor DOF1 tagged with HA[14] were co-transfected into protoplasts, and the proteins were labelled with [^{35}S]Met in the presence or absence of 10 mM glucose or 3-OMG. Neither glucose nor 3-OMG affected the labelling patterns of total cytoplasmic and nuclear proteins (Fig. 2a), or the total amount of protein synthesis (data not shown). However, the amount of EIN3, but not DOF1, was specifically diminished by glucose but not by 3-OMG (Fig. 2b).

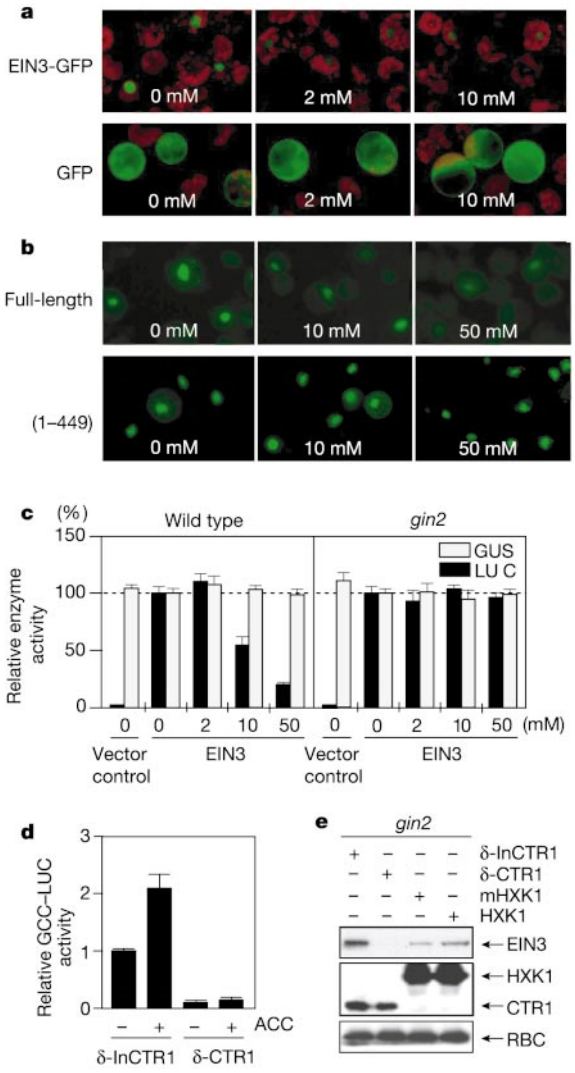

Figure 3 Differential glucose and ethylene signalling. **a**, Nuclear accumulation of EIN3–GFP in maize protoplasts. The red autofluorescence is from chlorophyll. The glucose concentration is shown. **b**, Nuclear accumulation of EIN3–GFP in *Arabidopsis* protoplasts. The GFP-tagged N terminus of EIN3 (residues 1–449) is insensitive to glucose. **c**, Glucose repression of EIN3 activity is abolished in the *gin2* mutant protoplasts. LUC, *EBS–LUC*; GUS, *UBQ10–GUS*. **d**, Ethylene and ACC activation of *GCC–LUC* is abolished by CTR1. δ-CTR1, constitutively active CTR1; δ-InCTR1, kinase mutant of CTR1. **e**, EIN3 accumulation is reduced by CTR1 and HXK1 in the *gin2* mutant protoplasts. mHXK1, catalytically inactive HXK1; RBC, Rubisco.

Thus, glucose might accelerate the degradation of EIN3, thereby reducing the activation of *EBS–LUC*.

To monitor the degradation of EIN3 directly *in vivo*, we incubated the transfected and labelled protoplasts in the presence of 10 mM glucose or 3-OMG for 1, 3 and 6 h. The amount of EIN3 protein was determined by immunoprecipitation. It was evident that EIN3 degradation was much faster in the presence of glucose than in the presence of 3-OMG or in the water control (Fig. 2c, d). To determine the nature of the proteolytic machinery involved in EIN3 degradation, we established a cell-free degradation assay[19,20] and tested the ability of different protease inhibitors to stabilize EIN3. Purified EIN3 was completely degraded within 60 min when incubated with maize protoplast cell extract (Fig. 2e). MG132, a potent inhibitor of the 26S proteasome[19–21], blocked degradation of EIN3 (Fig. 2f). Two other proteasome inhibitors, ALLM and ALLN[21], similarly hampered EIN3 degradation, whereas the general protease inhibitor leupeptin did not.

To investigate the subcellular localization of EIN3 in living cells, we fused EIN3 to jellyfish GFP for easy visualization[14,15,17]. EIN3–GFP accumulated in the nucleus (Fig. 3a), consistent with the location of an EIN3–GUS fusion protein[6]. Glucose decreased the accumulation of EIN3–GFP in the nucleus (Fig. 3a), similar to its effect on Myc-tagged EIN3 (Fig. 2b–d). The stability of GFP alone was not affected by glucose (Fig. 3a).

To see whether a similar glucose response might occur in another plant system, we examined the expression of two EIN3–GFP proteins in the presence or absence of glucose by using an *Arabidopsis* mesophyll protoplast transient assay[15]. As observed in maize protoplasts, GFP-tagged full-length EIN3 was detected in the nucleus but diminished with increasing concentrations of glucose (Fig. 3b). A GFP-tagged amino-terminal fragment (residues 1–449) of EIN3 accumulated in the nucleus but was insensitive to glucose (Fig. 3b), suggesting that the carboxy terminus of EIN3 is involved in the glucose response.

We also showed that the EIN3-dependent expression of *EBS–LUC* was repressed by glucose in *Arabidopsis* protoplasts (Fig. 3c). To confirm the requirement of AtHXK1 in the glucose-mediated repression of EIN3 activity, a transient expression analysis was carried out with protoplasts of the *Arabidopsis* AtHXK1-null mutant *gin2* (refs 3, 8). As shown in maize protoplasts (Fig. 1d), AtHXK1 also controlled EIN3-dependent expression of *EBS–LUC* in *Arabidopsis* protoplasts (Fig. 3c). Thus, the regulatory mechanism mediating glucose-dependent degradation of EIN3 is probably conserved in maize and *Arabidopsis*.

Arabidopsis protoplasts, however, seemed to be less sensitive to glucose because a higher glucose concentration was required to reach the effect observed in maize protoplasts (Figs 1c and 3a–c). Because *Arabidopsis* protoplasts were isolated from 3–4-week-old mature leaves, in which endogenous ethylene signalling is more active than in seedlings, these cells are probably more resistant to glucose than are maize cells isolated from the leaves of 11-day-old seedlings[11,13].

To examine ethylene responses in *Arabidopsis* protoplasts, we tested a *LUC* reporter gene controlled by a well-characterized ethylene responsive enhancer GCC (*GCC–LUC*)[22]. As expected, *GCC–LUC* was active in *Arabidopsis* protoplasts. Addition of the immediate ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) further enhanced its activity, suggesting that these cells are responsive to ethylene (Fig. 3d). To provide further evidence that *GCC–LUC* activity is due to ethylene signalling in protoplasts, we introduced a constitutively active CTR1 variant (ref. 23), which has been identified as a key negative regulator of ethylene signalling[24]. Active CTR1 abolished *GCC–LUC* activity in *Arabidopsis* protoplasts by preventing the accumulation of EIN3 in wild-type and *gin2* protoplasts (Fig. 3d, e). Catalytically active or inactive AtHXK1 (ref. 8) was effective in diminishing EIN3 accumulation in *gin2* protoplasts (Fig. 3e). Thus, EIN3 is a common target of both the

ethylene and the glucose signalling pathways, which are uncoupled from glucose metabolism.

To investigate the link between the stability of EIN3 and the phenotypes associated with glucose and ethylene signalling *in planta*, we generated transgenic *Arabidopsis* plants expressing Flag-tagged EIN3 under the control of a constitutive 35S promoter[15]. The typical ethylene-induced triple response in dark-grown *Arabidopsis* seedlings was examined using ACC[4,6,18]. The *35S::EIN3–FLAG* seedlings showed a much stronger triple response than did wild-type seedlings, indicating that the EIN3–Flag protein is active in mediating ethylene responses. The *ein3* mutant[6] was insensitive to ACC under the same assay conditions (Fig. 4a). Although *EIN3* transcripts from both the endogenous gene and the transgene were present constantly (Fig. 4b), EIN3–Flag was detected only with ACC treatment, suggesting that ethylene enhanced the accumulation and/or stability of EIN3 (Fig. 4c).

To ensure that EIN3–Flag was regulated in the same way as endogenous EIN3, an EIN3-specific antibody was raised and used to detect ACC-dependent EIN3 accumulation (Fig. 4c). *35S::EIN3–FLAG* transgenic seedlings were also more resistant to glucose-induced developmental arrest[4] than were wild type. By contrast, the *ein3* mutant was much more sensitive to glucose than were wild-type seedlings. All plants grew similarly on MS (Murashige–Skoog) plates containing mannitol (2–6%), supporting the idea that EIN3 mediates specific responses to glucose and ethylene but not osmotic stress (Fig. 4d).

To connect these cellular observations to the whole plant, we monitored the degradation of EIN3–Flag in transgenic *Arabidopsis* seedlings in the presence of glucose or ethylene. Seedlings germinated on MS plates containing ACC were incubated in liquid MS medium containing 6% glucose or 6% mannitol and cycloheximide to block *de novo* protein synthesis. Immunoblot analysis showed that EIN3 degradation proceeded much faster in glucose-treated seedlings than in mannitol- or water-treated seedlings (Fig. 4e). In addition, a proteasome-specific inhibitor, MG132, blocked the glucose-enhanced degradation of EIN3 (Fig. 4f). We also found that the presence of ACC delayed EIN3 degradation (Fig. 4g).

Sugars have been known for decades to influence plant growth and development profoundly, and the most prevailing view has been that sugars simply provide a source of energy and carbon. But extensive genetic analyses of sugar signalling mutants showing *gin* and *glo* phenotypes have identified an unexpectedly intimate connection between the glucose and plant hormone signalling pathways in the model plant *Arabidopsis*[2–5]. Of these findings, an antagonistic interaction between glucose and the plant stress hormone ethylene has been the least expected[4]. Our results now identify a previously unknown glucose signalling mechanism involving degradation of the EIN3 transcription factor, a well-established key regulator of ethylene signalling[6,7]. This provides the first molecular link in the plant signalling network that connects seemingly unrelated and distinct signal transduction pathways.

Because the acceleration of EIN3 degradation by glucose was observed in maize and *Arabidopsis*, glucose-dependent protein degradation might be a conserved mechanism for controlling growth and development in plants. Under physiological conditions, endogenous glucose signals probably promote growth by antagonizing stress hormone ethylene signalling and potentiating growth hormone auxin signalling[8]. In both plants and animals, monitoring endogenous glucose concentrations could have a central regulatory role in coordinating nutrient metabolism and hormone synthesis and signalling, which are also influenced by environmental conditions such as light, temperature and stress in plants.

The regulation of protein stability has recently emerged as a key mechanism that controls photomorphogenesis[20] and plant hor-

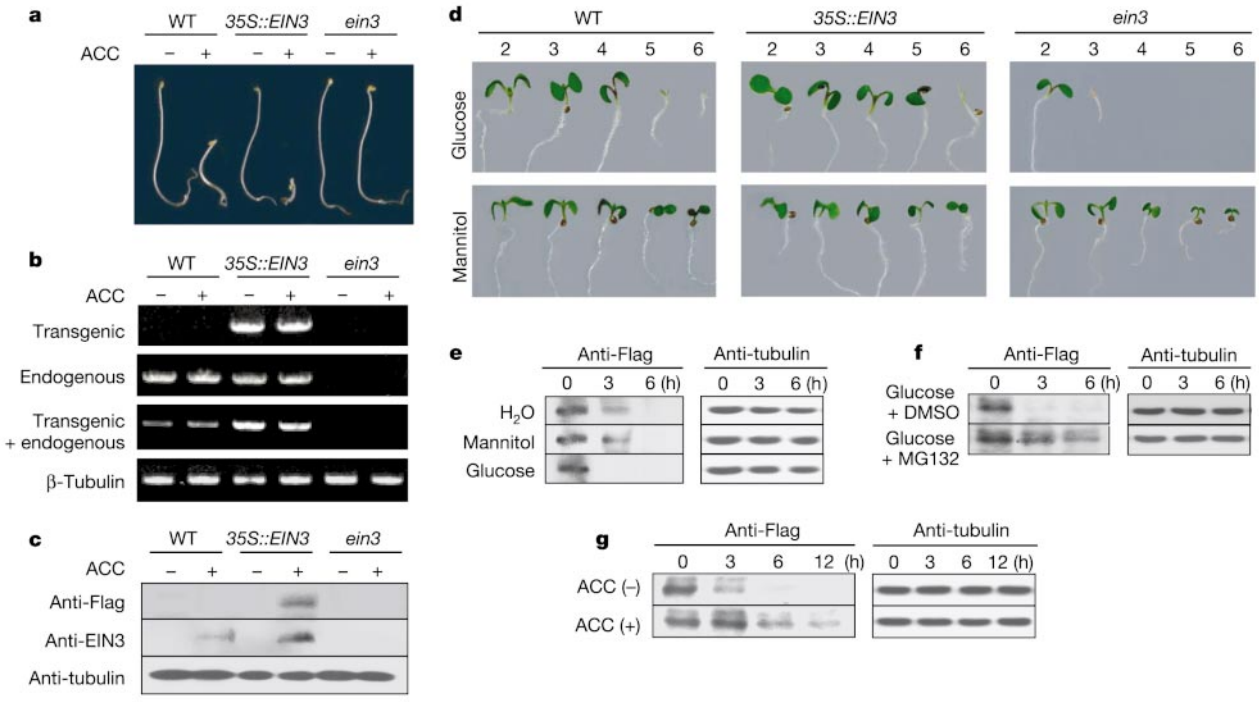

Figure 4 Opposite effects of ethylene and glucose on EIN3 stability in *Arabidopsis*. **a**, Phenotypic analyses of EIN3 transgenic plants. Dark-grown (5 d) wild-type (WT), transgenic (*35S::EIN3*) and *ein3* seedlings were treated without or with 20 μM ACC. **b**, Analysis of *EIN3* transcripts by PCR with reverse transcription. **c**, Immunoblot analysis of EIN3 protein. **d**, Glucose sensitivity assay. Seedlings were grown under constant light for 6 d. **e**, Glucose-dependent degradation of EIN3. Seedlings were treated with 100 μM cycloheximide plus H_2O, 6% mannitol or glucose for the indicated periods. **f**, MG132 inhibits glucose-dependent EIN3 degradation. Seedlings were treated with 100 μM cycloheximide plus 6% glucose and either dimethylsulphoxide (DMSO) or 20 μM MG132. **g**, ACC enhances EIN3 stability. Seedlings were treated with 100 μM cycloheximide plus 20 μM ACC.

mone signalling, and that mediates auxin[25], abscisic acid[26], gibberellin[27] and brassinosteroid[28] responses. Different ubiquitin protein ligases provide some specificity to direct diverse signalling molecules to the proteasome and signalosome[20,25,29]. It will be interesting to elucidate the precise pathways that link the glucose sensor AtHXK1 to the degradation of EIN3 and mediate the ethylene-enhanced EIN3 stability. Glucose has a profound influence on many essential processes in organisms as diverse as *Escherichia coli*, yeast, mammals and plants, but the complexity of glucose signalling mechanisms in multicellular animals and plants has been recognized only now[1,3]. Studies of glucose signal transduction in plants may provide insight into glucose signalling mechanisms that are conserved in eukaryotes from yeast to mammals. □

Methods

Effector and reporter constructs

To construct the inducer plasmid, we inserted a chimaeric gene encoding the LexA DNA-binding domain, the nuclear localization signal of the SV40 antigen, the transcriptional activation domain of VP16, and the glucocorticoid receptor (GR) domain[12] between the constitutive 35SC4PPDK promoter (a derivative of the 35S promoter) and the NOS terminator in a general plant expression vector[13]. For dexamethasone-inducible expression of EIN3, the EIN3 cDNA[6] was fused to a promoter containing four or eight copies of the LexA-binding site. For constitutive expression of EIN3 and EIL1, the respective cDNAs[6] were fused to sequence encoding the Myc epitope and inserted into the general plant expression vector[13]. The construct expressing constitutively active CTR1 (amino acids 544–799) has been described[23]. The inactive CTR1 mutant contained a point mutation (Lys579Met) generated by site-directed mutagenesis. The catalytically inactive AtHXK1 (S177A) has been described[8]. A control reporter construct (*NBS–LUC*) was generated by fusing the 35S minimal promoter to the firefly *LUC* gene without any enhancers. We generated the *EBS–LUC* and *GCC–LUC* reporter constructs by inserting four copies each of the EIN3-binding sequence[7,16] and the GCC-box enhancer sequence[22] in front of the 35S minimal promoter.

Protoplast transfection and reporter enzyme assays

Transfection of maize and *Arabidopsis* protoplasts was done as described[11,15]. As internal controls, we used *35S–GUS* or GUS reporter gene fused to the maize or the *Arabidopsis* ubiquitin promoter (*UBI–GUS* and *UBI10–GUS*)[13,14,16]. The reporter enzyme assays were done as described[11,15]. Data from duplicate or triplicate samples are shown with error bars. We carried out all reporter enzyme assays at least twice and obtained similar results.

Protoplast *in vivo* labelling and immunoprecipitation analysis

Transfected maize protoplasts were incubated for 1 h for mRNA accumulation before labelling with the [^{35}S]Met/Cys EXPRESS Protein Labeling Mix (NEN) for 5 h. Nuclear and cytoplasmic fractions were separated by treating the labelled protoplasts with 1% triton X-100, followed by centrifugation as described[13]. The amount of radioactivity in each sample was verified with a BAS-1000 Bio-imaging Analyzer (Fuji Film). For degradation analysis, transfected protoplasts were labelled for 3 h and then incubated in the presence of a 1,000-fold excess of unlabelled methione and cysteine. Epitope-tagged proteins were immunoprecipitated with an anti-Myc or anti-HA antibody as described[23], resolved on 12% SDS–PAGE gels, and analysed with the BAS-1000 analyser. For protein immunoblot analyses, we used an anti-Rubisco antibody[30], an anti-tubulin antibody (Sigma), a peroxidase-conjugated anti-Flag antibody (Sigma), an anti-HA antibody (Roche), and an anti-EIN3 antibody (a rabbit polyclonal antibody purified with recombinant EIN3 protein expressed from *E. coli*).

GFP fusions and fluorescence microscopy

We produced an expression vector for the GFP fusion protein by replacing the Myc tag with the GFP sequence in the EIN3–Myc vector. The sequence for the N terminus of EIN3 was amplified by polymerase chain reaction (PCR). GFP fluorescence was observed with a TE200 fluorescent microscope (Nikon) or a CK40 microscope (Olympus) equipped with a fluorescence condenser.

Cell-free degradation assay

^{35}S-labelled EIN3–Myc protein was produced in maize protoplasts, purified by immunoprecipitation and then used as a substrate. We prepared cell lysate by grinding maize protoplasts in a buffer supporting proteasome activity[20]. Degradation reactions were started by mixing the resin trapping the EIN3 or GFP (data not shown) substrate with the cell lysate, and stopped by adding a volume of 2× SDS loading buffer. The samples were separated on a 12% SDS–PAGE gel and analysed with the BAS-1000 analyser.

Transgenic plant analysis

Arabidopsis transgenic plants expressing EIN3–Flag under the control of the 35SC4PPDK promoter were generated by the floral dip method as described[5,8,15]. All phenotypic and immunoblot analyses were done using T3 homozygous seeds. We obtained similar results with two independent transgenic lines. To detect endogenous and Flag-tagged EIN3, proteins were extracted from 20 seedlings by grinding with 50 μl of 2× SDS loading buffer. For protein degradation analyses, T3 seeds were germinated on MS plates with 20 μM ACC for 5 d in continuous darkness. The seedlings were then incubated in liquid MS medium supplemented with 100 μM cycloheximide plus the indicated compounds. Proteins extracted from five seedlings by grinding with 20 μl of the 2× SDS-loading buffer were then analysed with an anti-Flag antibody.

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