Ethylene Regulates Monomeric GTP-Binding Protein Gene Expression and Activity in Arabidopsis[1]

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Ethylene rapidly and transiently up-regulates the activity of several monomeric GTP-binding proteins (monomeric G proteins) in leaves of Arabidopsis as determined by two-dimensional gel electrophoresis and autoradiographic analyses. The activation is suppressed by the receptor-directed inhibitor 1-methylcyclopropene. In the *etr1-1* mutant, constitutive activity of all the monomeric G proteins activated by ethylene is down-regulated relative to wild type, and ethylene treatment has no effect on the levels of activity. Conversely, in the *ctr1-1* mutant, several of the monomeric G proteins activated by ethylene are constitutively up-regulated. However, the activation profile of *ctr1-1* does not exactly mimic that of ethylene-treated wild type. Biochemical and molecular evidence suggested that some of these monomeric G proteins are of the Rab class. Expression of the genes for a number of monomeric G proteins in response to ethylene was investigated by reverse transcriptase-PCR. *Rab8* and *Ara3* expression was increased within 10 min of ethylene treatment, although levels fell back significantly by 40 min. In the *etr1-1* mutant, expression of *Rab8* was lower than wild type and unaffected by ethylene; in *ctr1-1*, expression of *Rab8* was much higher than wild type and comparable with that seen in ethylene treatments. Expression in *ctr1-1* was also unaffected by ethylene. Thus, the data indicate a role for monomeric G proteins in ethylene signal transduction.

Mutagenic analyses in Arabidopsis have made great inroads into unraveling the perception and transduction of the hormone ethylene. The ethylene signal transduction chain in Arabidopsis as presently conceived consists of five partially functionally redundant receptors (Bleecker et al., 1988; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998); a protein kinase, CTR1 (Kieber et al., 1993); a possible ion transporter, EIN2 (Alonso et al., 1999); and transcription factors. Two classes of ethylene-responsive transcription factors have been extensively characterized: the EIL series (Chao et al., 1997; Solano et al., 1998), which also appear to exhibit partial functional redundancy, at least in tomatoes (*Lycopersicon esculentum*; Tieman et al., 2001) and ethylene response element-binding proteins (Solano et al., 1998). The receptors appear to regulate the signaling chain negatively; that is, they are active in the absence of ligand and inactive when binding it (Hua and Meyerowitz, 1998; Hirayama et al., 1999). Moreover, both dominant (Bleecker et al., 1988; Hua et al., 1995, 1998; Sakai et al., 1998) and recessive (Hua and Meyerowitz, 1998) receptor mutants have been produced. The former are insensitive to ethylene, e.g. *etr1-1* (Hall et al., 1999), and in these cases, the receptor is presumably "locked" into its active state. In the recessive mutants, severely truncated specific receptor proteins appear to be made and in these cases can be said to correspond to the "inactive" state. These mutants have normal phenotypes and, with one exception (*etr1-6*), show wild-type responses to ethylene, presumably due to functional redundancy (Hua and Meyerowitz, 1998). In line with this hypothesis, crosses between two of the recessive mutants (*etr1-6* and *ein4-4*) show some constitutitive "ethylene-treated" developmental characteristics, a triple cross (*etr1-6,etr2-3,ein4-4*) shows a strong "ethylene-treated" phenotype, comparable with *ctr1-1* (the mutant wherein the lesion is in the gene for the CTR1 protein), and a quadruple cross (*etr1-6,etr2-3,ein4-4,ers2-3*) shows an extreme phenotype, much more severe than *ctr1-1*.

Receptor function has been shown to be dependent on Ran 1 and Ran2 (response to antagonist Menkes/Wilson disease-related copper transporters; Hirayama et al., 1999), which are located in Golgi/post-Golgi bodies in mammalian systems. A model has been proposed where Ran1/Ran2 are instrumental in the incorporation of copper into ethylene receptors that are trafficking from the Golgi to plasmalemma within vesicles (Woeste and Kieber, 2000).

Placed downstream of the ethylene receptors is the *ctr1-1* mutant where the lesion is in a gene that exhibits homology to Raf class mitogen-activated protein kinase kinase kinases (MAP3K; Kieber et al., 1993). The recessive *ctr1-1* mutant has an "ethylene-

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treated" phenotype (Kieber et al., 1993), which has been taken to mean that this component, when active, represses ethylene effects (Kieber et al., 1993). This is in agreement with the negative regulatory effects of the receptors, one or more of which activate CTR1 in their active state. Activation ceases when the receptor(s) become inactive on binding ethylene. However, as the other downstream effectors EIN2, the EIL series and the ethylene response element-binding proteins are positively regulated by ethylene, it is unclear how this is achieved if CTR1 is the only downstream effector.

We have now demonstrated both in Arabidopsis (Novikova et al., 2000) and in peas (*Pisum sativum*; Hall et al., 2001) that ethylene rapidly up-regulates the activity of protein kinase(s) of the MAP kinase (MAPK) type; in Arabidopsis at least, this appears to be due to activation of existing enzymes. Equally, Kumar and Klessig (2000) have shown that in tobacco (*Nicotiana tabacum*), treatment with the ethylene precursor aminocyclopropane carboxylic acid leads to a transient increase in MAPK activity.

From these data, we have argued for the existence of a separate ethylene-up-regulated MAPK cascade, somehow antagonistic to that controlled by CTR1 (Hall et al., 2001). A precedent for this exists for auxin signaling, because whereas the hormone can up-regulate MAPK activity in Arabidopsis (Mockaitis and Howell, 2000), transient expression of the MAP3K NPK1 antagonizes auxin effects (Kovtun et al., 1998). If such an antagonistic cascade exists for ethylene, then the question arises as to how it is controlled. Monomeric GTP-binding proteins (monomeric G proteins) are ubiquitous components of signaling systems in animals (Bos, 2000) and yeast (Schmidt and Hall, 1998), and one group of their effectors is MAP3K (Daum et al., 1994) and, hence, MAPK cascades, although there are many others. A large number of genes for monomeric G proteins have been isolated from plants and, based on homology to mammalian genes, classified into Ran (nuclear located; Görlich and Kutay, 1999; Smith and Raikhel, 1999), Rho, Rac, or Rab classes. Plant Rho monomeric G proteins, known as Yops (in tomato) and Rops (in Arabidopsis), are associated with regulating developmental events such as pollen tube elongation (Li et al., 1999), whereas plant Rac monomeric G proteins have a clear role in plant defense, being involved in cell death (Schiene et al., 2000), the generation of an oxidative burst in rice (*Oryza sativa*; Ono et al., 2001) and susceptibility to *Blumeria graminis* in barley (*Hordeum vulgare*; Schultheiss et al., 2002). In mammals, the large Rab class is involved with endosomal movement, including the regulation of vesicle trafficking between the Golgi bodies and the plasmalemma. A similar function has now been demonstrated for plant Rab proteins (designated "Ara" in Arabidopsis). Thus, Rab1 appears to regulate trafficking between the endoplasmic reticulum and Golgi apparatus (Batoko et al., 2000), and Ara6 and Ara7 have been shown to cycle between post-Golgi vesicles and the plasmalemma (Ueda et al., 2001).

Although phenotypic screens in Arabidopsis have yielded no mutants with lesions in monomeric G protein genes, monomeric G proteins have been shown to be transcriptionally up-regulated by ethylene in tomato (Loraine et al., 1996; Zegzouti et al., 1999). In this context, we have shown previously that in peas (Novikova et al., 1997) and in Arabidopsis (Novikova et al., 1999), ethylene up-regulates the activity of monomeric G proteins. Recent work in peas (Moshkov et al., 2003) indicates that several monomeric G proteins are so activated, that the effect of ethylene is very rapid (2 min), and that the response in some cases is bimodal (Moshkov et al., 2003), as in some animal systems (Foschi et al., 1997). Furthermore, *Rab1A* expression was induced with fruit ripening and after ethylene treatment in tomato (Loraine et al., 1996), and transgenic tomato plants containing antisense *Rab11* constructs exhibited abnormal phenotypes and reduced fruit softening (Li et al., 2001). Equally, we have also shown that in pea, some monomeric G proteins activated by ethylene are precipitated by antibodies to Rab8 (Moshkov et al., 2003).

This work prompted us to examine in more detail the effect of ethylene upon the activation and also the transcription of monomeric G proteins in Arabidopsis that, through the availability of suitable mutants, allowed us to integrate monomeric G protein action with the established ethylene signal transduction chain. Using two-dimensional electrophoresis, we observed that, as in peas, the activity of a large number of monomeric G proteins is rapidly up-regulated by ethylene. Moreover, in the *etr1-1* mu-

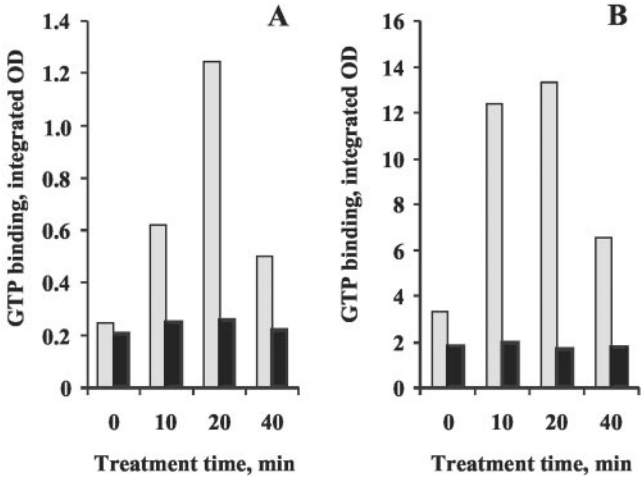

Figure 1. Effect of ethylene on activation of monomeric G proteins in 750 mM KCl (A) and Triton X-100 (B) fractions from Arabidopsis wild-type (□) and *etr1-1* (■) plants. After extraction, proteins were labeled with [α-^{32}P]GTP and subjected to SDS-PAGE followed by autoradiography. Experimental points are derived from scans of autoradiographs.

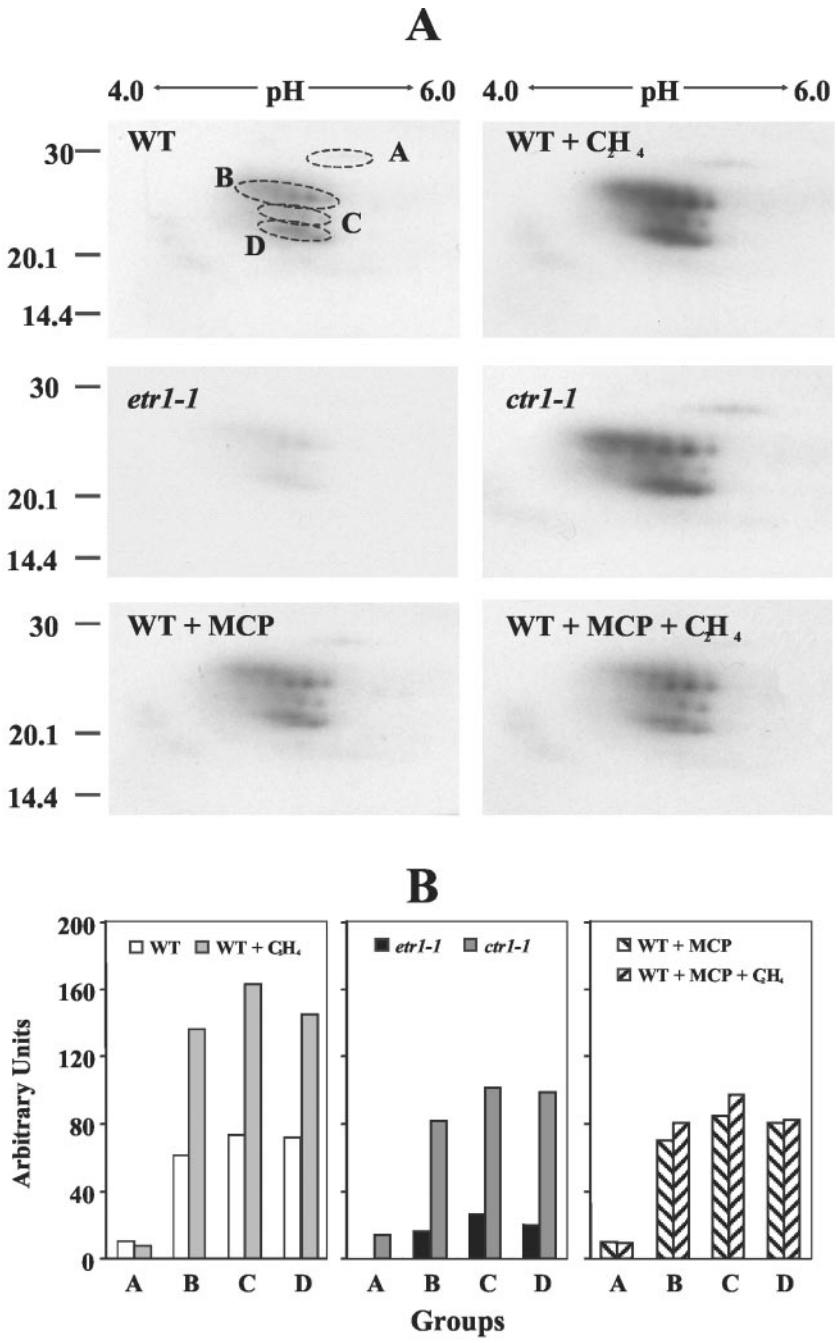

Figure 2. Separation of monomeric G protein components from Arabidopsis leaf membranes solubilized with Triton X-100 in two-dimensional PAGE. Components were grouped by their molecular masses (A), and GTP binding in groups was quantified by scanning of autoradiographs (B).

tant, the activities are constitutively down-regulated, and in *ctr1-1* many are markedly up-regulated. Studies using reverse transcriptase (RT)-PCR of a number of genes for monomeric G proteins showed that transcription of the *Rab8* and *Ara3* genes was rapidly and transiently up-regulated by ethylene and that their transcription was perturbed in ethylene signaling mutants. To our knowledge, these data represent the first non-correlative evidence for monomeric G protein action in ethylene-associated events and is suggestive of a role for the Rab-class monomeric G proteins.

RESULTS

The Activity of Multiple Monomeric GTP-Binding Proteins Is Increased after Ethylene Treatment and Is Regulated by Components of the Ethylene Signal Transduction Chain.

Proteins were extracted from light membrane fractions from Arabidopsis wild type or *etr1-1* mutant exposed to 1 μL L^{-1} ethylene for up to 40 min, using 750 mM KCl followed by Triton X-100 (representing extrinsic and integral protein components, respec-

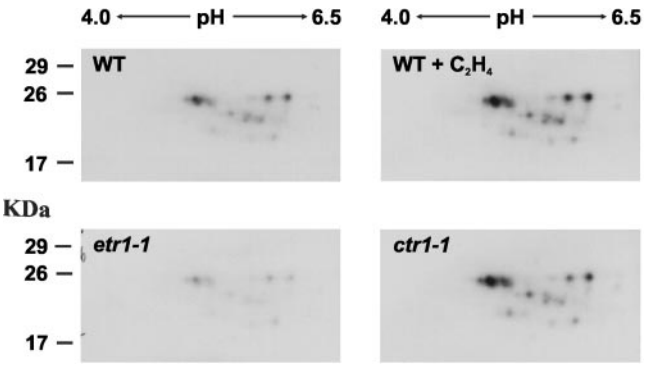

Figure 3. Separation of monomeric G protein components from Arabidopsis leaf membranes extracted with 750 mM KCl in two-dimensional PAGE.

tively) and fractionated by one-dimensional PAGE. Specific GTP binding was assessed by probing with [α-^{32}P]GTP in the presence and absence of excess GTP. No specific GTP binding was observed at molecular masses over 30 kD; hence, heterotrimeric G proteins do not represent a significant component in these preparations. No nonspecific ethylene binding was observed between 20 and 30 kD in this or in subsequent two-dimensional separations. The relative densities of the GTP-binding components between 20 and 30 kD over the time course are shown in Figure 1. In wild type in both fractions, activity increased markedly (2–4-fold) within the first 10 min of ethylene treatment, reached a maximum at 20 min and fell back significantly by 40 min. In *etr1-1*, activity was lower than in untreated wild type and was unaffected by ethylene. It should be noted that around 75% of the total activity is found in the Triton X-100 fractions.

Two-dimensional PAGE separations of comparable Triton X-100 samples are shown in Figure 2A. Because of the high hydrophobicity of the proteins, individual components do not in general appear as distinct spots so they were arbitrarily grouped by molecular mass and scanned for activity (Fig. 2B). The up-regulation of GTP binding is observable in all but one of the groups and to about the same extent. Pretreatment for 2 h with the receptor-directed inhibitor 1-methylcyclopropene (MCP) led to some up-regulation in three of the groupings (as we have observed in peas; Moshkov et al., 2002), but ethylene added after this time did not result in further up-regulation. Similar effects were observed in the KCl fraction (results not shown). Ethylene accelerates the rate of senescence in excised Arabidopsis leaves (Novikova et al., 1999), and studies in the present work showed that MCP alone delays senescence significantly and completely nullifies the effect of ethylene. Percentages for remaining chlorophyll in detached leaves after 72 h of treatment were: control, 33%; ethylene, 21%; MCP, 58%; and MCP plus ethylene, 57%. In similar experiments, the constitutive levels of GTP binding in *etr1-1* and *ctr1-1* were investigated (Fig. 2). In *etr1-1*, all the groupings were down-regulated relative to wild type. However, it should be borne in mind that the wild type is not zero ethylene and that Arabidopsis produces the hormone endogenously at a relatively high rate (Sanders et al., 1991a). Hence, some of the activity seen in wild type may be due to endogenous ligand, and the low levels in *etr1-1* likely represent the equivalent of zero ethylene because the mutated ETR1 protein does not bind ethylene (Schaller and Bleecker, 1995).

Two-dimensional PAGE separations of KCl fractions are shown in Figure 3. The overall picture is the same as that observed in Triton extracts: up-regulation by ethylene and constitutive down-regulation and up-regulation in *etr1-1* and in *ctr1-1*, respectively. However, it was possible to identify 17 separate components on these gels (Fig. 4A), and 10

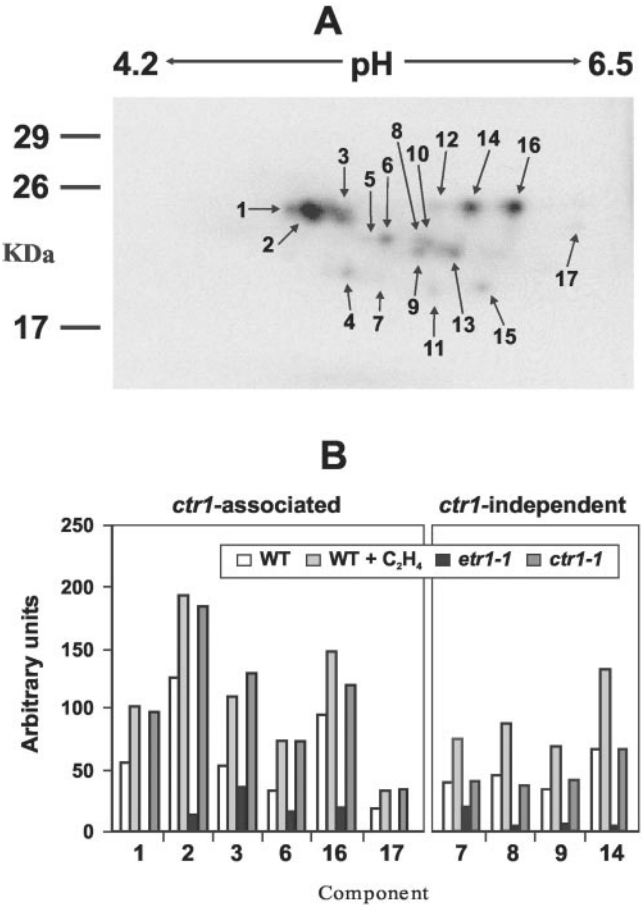

Figure 4. Quantification of GTP binding to Arabidopsis monomeric G proteins in 750 mM KCl-extracted protein preparations. A, Detected GTP-binding components in preparations from *ctr1-1* were designated from 1 to 17. B, GTP binding was quantified for 10 components that could be identified in wild type untreated (white squares), wild type ethylene treated (light-gray squares), and in Arabidopsis mutants *etr1-1* (black squares) and *ctr1-1* (dark-gray squares). Results were divided as "*ctr1* associated," i.e. GTP binding in *ctr1-1* was equivalent to that observed in ethylene-treated wild-type plants, or "*ctr1* independent," where no increase in GTP binding over untreated levels was observed in *ctr1-1*.

Table I. *Deduced molecular masses and apparent pIs of GTP-binding components KCl extracted from Arabidopsis* membranes

Component	pI	Molecular Mass	Component	pI	M_r
		kD			
1	4.9	24.3	10	5.6	21.9
2	5.0	24.1	11	5.6	19.1
3	5.1	24.1	12	5.6	24.6
4	5.2	20.0	13	5.7	21.7
5	5.2	22.5	14	5.7	24.7
6	5.3	22.5	15	5.8	19.3
7	5.3	20.0	16	5.9	24.9
8	5.5	22.4	17	6.2	23.5
9	5.5	21.7	–	–	–

of these were sufficiently distinct to allow the extent of GTP binding to be quantified. Up-regulation by ethylene was observed in all cases and, as with Triton, activity in *etr1-1* was much lower than in wild type. Interestingly, although in six of the components constitutive activity was up-regulated in *ctr1-1* to levels comparable with those in ethylene-treated wild type, in four cases only ethylene caused an activation and levels in *ctr1-1* were similar to those in wild-type controls (Fig. 4B).

Although it has not proved possible so far to identify individual components precisely, nevertheless, calculation of the pIs and molecular masses of the components on the gels for KCl fractions (Table I) allows comparisons with data on monomeric G proteins derived from the Arabidopsis database using the approach of Bjellqvist et al. (1993; Table II). Thus, the highly distinct pI ranges and clustering of predicted and measured molecular masses for both Rac and Rho class monomeric G proteins suggest that the ethylene-activated proteins are not of these types. In addition, given the methods used to isolate the light membranes, it is unlikely that nuclear-located Ran class monomeric G proteins would be present. Hence, the two-dimensional PAGE results suggest the detection of Rab-type proteins.

Expression of Genes for Selected Monomeric G Proteins Is Up-Regulated by Ethylene

In moving toward characterizing which monomeric G proteins were influenced by ethylene, we hypothesized that some of the elevated activities observed in Figures 2 to and 4 were derived from de novo gene expression. A monomeric G protein gene (*ER43*), which was rapidly induced after ethylene treatment and showed homology to a monomeric G protein from pea, has been previously identified from tomato (Zegzouti et al., 1999). Our database searches revealed that these sequences are most homologous to Rab8/Ara3 proteins found in Arabidopsis. Distinctive motifs such as GTP-binding sites, GTPase, and isoprenylation domains are conserved in all but one of the Rab8 class proteins so far noted in plants. Membrane-interacting regions were tentatively identified only within Ara3 and Rab8 (amino acids 36–56) of all the Rab-class MGBPs. Such a region was also detected with Rac2 and Rop4 but in a different position in the protein (Fig. 5A). Phylogenetic analyses of monomeric G protein amino acid sequences, focusing especially on the Rab class, demonstrated that the Rab8 sequences formed a discrete and conserved grouping (Fig. 5B). Thus, when examining the possible transcriptional regulation by ethylene of monomeric G protein genes, most targets were of the Rab class (*Rab8*, *Ara2*, *Ara3*, *Ara4*, and *Ara5*). However, representative examples of the more genetically distant Rac (*Rac2*) and Rho (*Rop4*) classes were also examined. mRNA was isolated from Arabidopsis wild-type mature rosette leaves treated with 1 μL L^{-1} ethylene for up to 40 min, and first strand cDNA was synthesized and quantified. Monomeric G protein transcript abundance was determined by RT-PCR (Fig. 6). Monomeric G protein gene expression was equilibrated for sample variability by comparison with the expression of cinnamyl alcohol dehydrogenase (CAD), and the total expression (Fig. 7A) and the fold ethylene inducibility of each monomeric G protein (Fig. 7B) were determined. These indicated that only *Ara3* and particularly *Rab8* exhibited ethylene-inducible expression. *Ara5* displayed the highest level of expression, although no regulation by ethylene was observed. *Ara3/Rab8* expression returned to baseline levels by 40 min of ethylene treatment and, interestingly, the expression of genes for several monomeric G proteins, including *Rac2*, was suppressed at the later time points.

Table II. *Biochemical characteristics of Arabidopsis* monomeric G proteins

Predicted M_rs and pIs were calculated using Compute pI/MW (http://www.expasy.ch/tools/pi_tool.html; Bjellqvist et al., 1993).

Class	*n*	M_r		pI	
		Median	Range	Median	Range
Rab	11	23,848	22,318–24,980	6.17	4.98–8.36
Ran	3	25,080	25,080–25,276	6.39	6.39–6.71
Rac	11	21,728	21,571–23,043	9.24	9.18–9.63
Rho	4	21,682	21,619–21,782	9.3	9.21–9.38

The *etr1* and *ctr1* Mutations Affect *Rab8* Gene Expression

In identical experiments to those described above, leaf material from the *etr1-1* and *ctr1-1* mutants was probed for expression of *Rab8*, and the results are shown in Figure 8. In *etr1-1*, expression was lower than that in wild type and was unaffected by ethylene. In contrast, expression in *ctr1-1* was much higher than that in wild type, but comparable with the levels seen in the latter after 10 min of ethylene treatment; again, expression was unaffected by ethylene.

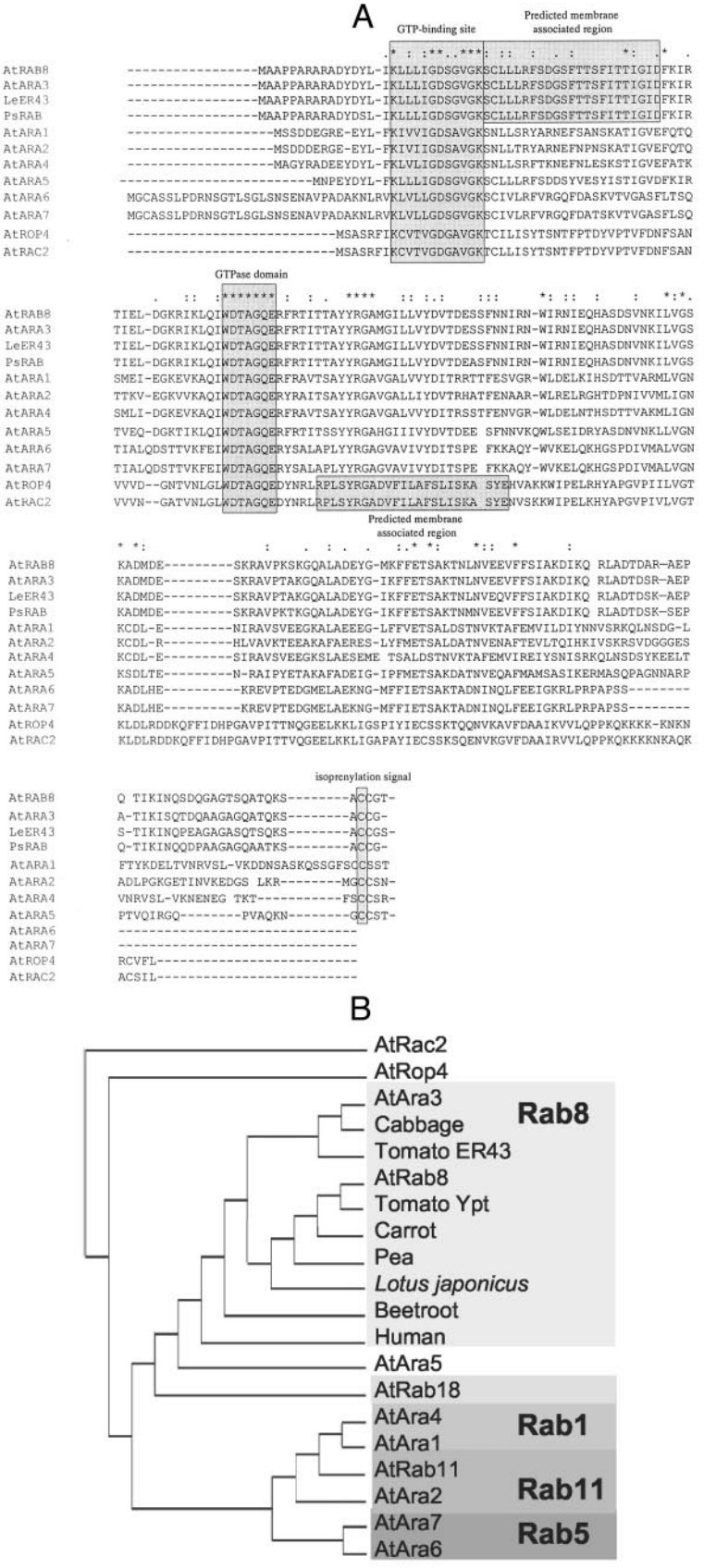

Figure 5. Amino acid homologies and phylogenetic relationship between Rab8 from a range of species to other monomeric G protein classes. A, Lineup of amino acid sequences from the Rab8 class (accession nos.: Arabidopsis, Rab8, T45901; Ara2, P28185; Ara3, P28186; Ara4, P28187; Ara5, P28188; Ara 6, BAB32953; and Ara7, BAB32669; tomato, ER43, AAD46405; and pea, S33531) and representatives of the Rac2 (accession no. AF107663.1) and Rho (Rop4, accession no. AAC78242) classes. Conserved motifs associated with GTP binding, GTPase activity, and isoprenylation are highlighted. Tentatively identified membrane-associating regions are also boxed. B, Phylogenetic relationships between monomeric G protein amino acid sequences using Arabidopsis AtRac2 (accession no. AF107663.1) as the outgroup. Rab8-class sequences from Arabidopsis (accession nos.: Rab8, T45901; and Ara3, P28186) with representatives from cabbage (*Brassica campestris*; accession no. T14405), tomato (accession nos.: ER43, AAD46405; and Ypt, S33900), carrot (*Daucus carota*; accession no. CAA04701), pea (accession no. S33531), *Lotus japonicus* (accession no. CAA98172), beetroot (*Beta vulgaris*; accession no. T14565), and human (*Homo sapiens*; accession no. B49647). Amino acid sequences for the Arabidopsis monomeric G proteins Rop4 (accession no. AAC78242), Ara5 (accession no. P28188), Rab18 (accession no. AAB61997), Ara4 (accession no. P28187), Ara1 (accession no. AY063847), Rab11 (accession no. AAL38821), Ara2 (accession no. P28185), Ara6 (accession no. BAB32953), and Ara7 (accession no. BAB32669) are also included.

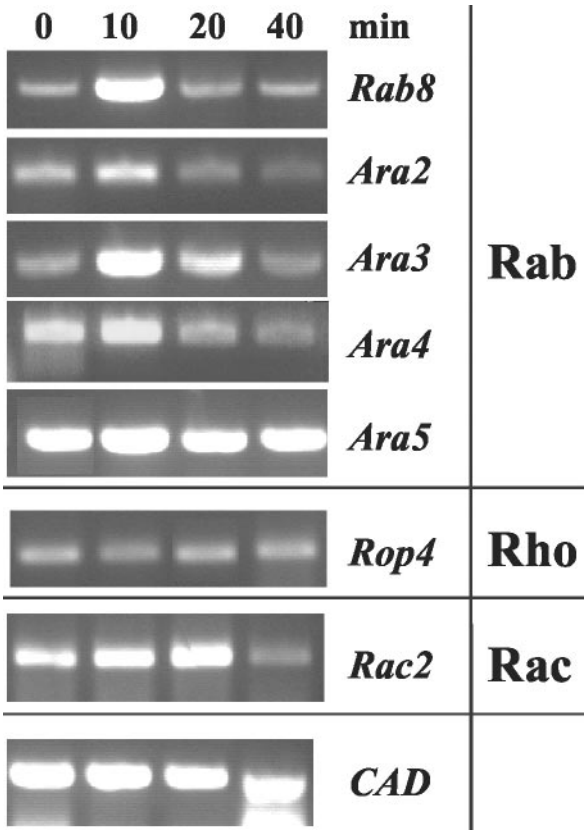

Figure 6. Transcriptional analysis of expression of monomeric G proteins after ethylene treatment. Arabidopsis plants were treated with ethylene for 0, 10, 20, and 40 min, and RNA was isolated from each of three Arabidopsis plants per time point. First strand cDNA was constructed from each plant. Specific oligonucleotide primers were used to detect transcript levels of *Rab8*, *Ara2*, *Ara3*, *Ara4*, *Ara5*, *Rop4*, and *Rac2* in 1 μg of first strand cDNA. Amplifications from each cDNA from each plant were repeated three times (n = 9), representative results of which are shown. Control amplifications were carried out using oligonucleotide primers to *CAD*.

DISCUSSION

The work described here shows that, as in peas (Moshkov et al., 2003), ethylene rapidly but transiently promotes GTP binding in a number of monomeric G proteins in Arabidopsis leaves. It should be noted that not all the components are necessarily separate monomeric G proteins. The proteins could represent products of different genes or isoforms of the same gene. In addition, it is well established that procedures before electrophoresis may modify proteins such that a single component can give rise to more than one spot (Celis and Gromov, 1999).

Although in *etr1-1* there is constitutive down-regulation, in *ctr1-1* constitutive activation is much higher than in wild type and comparable in part with wild type treated with ethylene. Expression of two genes, *Rab8* and *Ara3*, is also rapidly but transiently up-regulated by ethylene; in *etr1-1*, the constitutive expression of *Rab8* is low, whereas in *ctr1-1* it is much higher than in wild type; in neither case is expression affected by ethylene. We believe that these results lend further credence to the hypothesis that monomeric G proteins are involved in ethylene signal transduction. At present, it is not possible to distinguish the reason for the increased GTP-binding activities. However, some of the earliest effects are more likely to be due to activation of existing protein, whereas some of the later effects could be explained by either activation of existing protein or de novo synthesis. Although compared with the situation in animals and in yeast (Lazar et al., 1997; Rommel and Hafen, 1998; Shields et al., 2000), the evidence for a role for monomeric G proteins in plant growth and development is relatively sparse, there is increasing interest in these molecules. Thus, Li et al. (1999) demonstrated a role for Rop1 in pollen tube polarity and more recently Li et al. (2001) have shown that Rop-type proteins are involved in the regulation of a wide range of developmental events. Further, there are many examples where monomeric G proteins have been shown to play a role in plant defense (Schiene et al., 2000; Ono et al., 2001; Schultheiss et al., 2002). It is also clear from the studies of several workers that, as in animals (Mohrmann and van der Sluijs, 1999), Rab-type proteins are involved in vesicle trafficking (Batoko et al., 2000). The fact that the preparations used for these experiments are light membranes and, therefore, enriched in Golgi and endoplasmic reticulum tends to lend support for such a role. Previous work with peas (Novikova et al., 1997) indicated that there was no significant activation of monomeric G proteins in fractions enriched in plasmalemma. Given these widespread effects, it would be surprising if the mechanism of action of ethylene, and other plant hormones whose effects are pleiotropic did not involve monomeric G proteins.

In this connection, Zegzouti et al. (1999) have demonstrated that transcription of a gene for a Rab-class protein (ER43; Fig. 5) is transiently up-regulated by ethylene, and recent work by Lu et al. (2001) has shown that in tomato plants, expression of an antisense *Rab11* gene reduces fruit softening—a process long known to be associated with ethylene. We ourselves have demonstrated that in pea epicotyls, as in Arabidopsis, ethylene up-regulates the activities of several monomeric G proteins within 2 min and that the activation is transient but also bimodal. In some cases, transient unimodal activation is observed, whereas in others transient down-regulation occurs—the latter being reminiscent of some of the effects on transcription demonstrated in this work. The activations are abolished by the ethylene receptor-directed inhibitor MCP (Moshkov et al., 2003).

In a broader context, it seems likely, given the established signaling role of monomeric G proteins in animals and yeasts and their emerging roles in plants, that the effects of ethylene both on activation

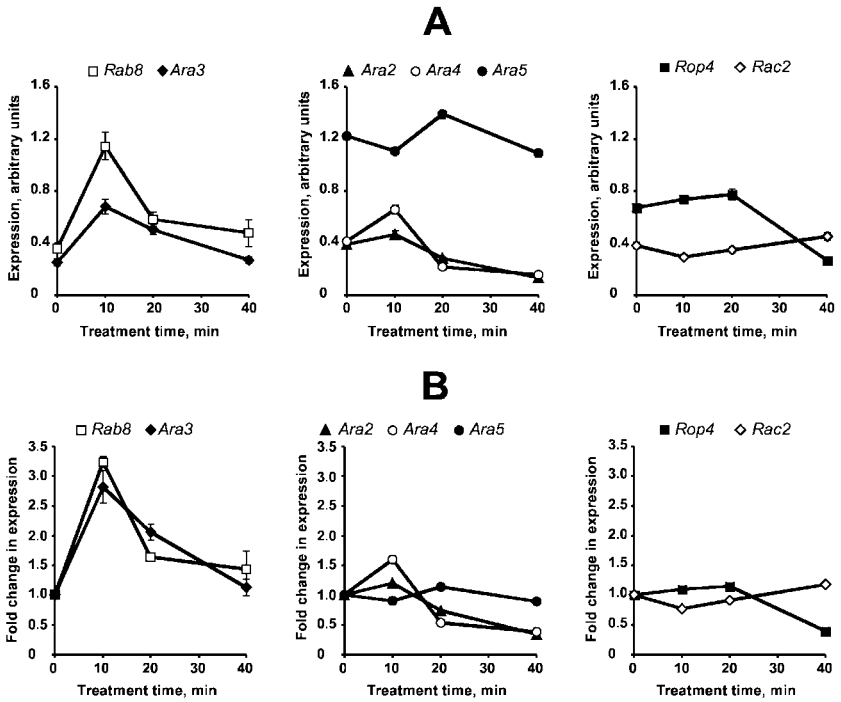

Figure 7. Quantification of monomeric G protein expression after ethylene treatment. RT-PCR-amplified bands (those shown in Fig. 6 and in eight repeat experiments) representing monomeric G proteins were quantified and equilibrated relative to the constitutive control, *CAD* levels in samples isolated from the same plant. Monomeric G protein expression is expressed either in terms of "absolute" levels relative to *CAD* (A) or fold "ethylene inducibility" relative to transcript accumulation at no ethylene treatment (B). Results are given as mean ($n = 9$) ± SE.

of these components as well as on transcription indicate a role for them in ethylene signaling. Five pieces of evidence argue strongly for such a role. First, the timing and kinetics. To our knowledge, the activation of some monomeric G proteins in response to ethylene in peas occurs more rapidly than any recorded biochemical effect of ethylene in intact tissue, but in the same time frame as some developmental responses—such as the inhibition of root growth (Warner and Leopold, 1971). The 4-fold activation shown to occur in 10 min in the present work is equally dramatic. The rapidity of the responses mirrors the kinetics in animal systems (Foschi et al., 1997) and is consistent with the behavior of a signaling molecule close to the site of perception. In this connection, in vivo studies of ethylene binding indicate the presence of receptor components in both peas and Arabidopsis having high rate constants of association (Sanders et al., 1991a, 1991b), suggesting that signal transduction would occur rapidly after binding of the ligand by the receptor. The same studies indicated components having very low rate constants of association/dissociation. ETR1 expressed in yeast shows a low rate constant of dissociation (Schaller and Bleecker, 1995), but calculations using the rate constant of dissociation and the K_D indicate a very high rate constant of association (A. Bleecker, personal communication). This implies that the receptors must exist in both kinetic forms since in a matter of minutes after exposure to ethylene only an infinitesimal proportion of slow associating sites would be occupied by ligand. It may also be significant that Rab proteins are associated with the endomembrane system (see Chavrier and Goud, 1999), which is also where both the bulk of ethylene binding is located (Evans et al., 1981, 1982; Schaller and Bleecker, 1995; Schaller et al., 1995) and where the ETR1 protein expressed in yeast also appears to be localized. Second, the bimodal activation in peas corresponds closely to that seen in some animal systems subjected to a continuous hormonal signal (Foschi et al., 1997). It is interesting that in such systems the first peak of activation corresponds to the initiation of MAPK cascades (which we have observed both in peas [Hall et al., 2001] and Arabidopsis [Novikova et al., 2000]). Third, the rapid activation of gene transcription for specific monomeric G proteins is reminiscent of work with auxins where both MAPK activation is observed (Mockaitis and Howell, 2000) and increased gene transcription for these signaling molecules (Mizoguchi et al., 1994). Fourth, the fact that in the ethylene-insensitive receptor mutant *etr1-1*, the monomeric G proteins activated by ethylene in wild type are constitutively down-regulated also suggests a relationship with ethylene signaling because such mutants do not respond to the hormone. The fact that transcription of the genes for a monomeric G protein—*Rab8*, which appears to be activated in both peas and Arabidopsis—is down-regulated in *etr1-1* and up-regulated in *ctr1-1* also lends support to the hypothesis. Equally, the constitutive up-regulation of a majority of the monomeric G proteins activated by ethylene seen in *ctr1-1*, a mutant showing an "ethylene-treated" phenotype, is also suggestive. Fifth, the fact that not all of the monomeric G proteins activated by ethylene are up-regulated in *ctr1-1* is consistent with the results of Hua and Meyerowitz (1998) using crosses of recessive receptor mutants, where a quadruple cross

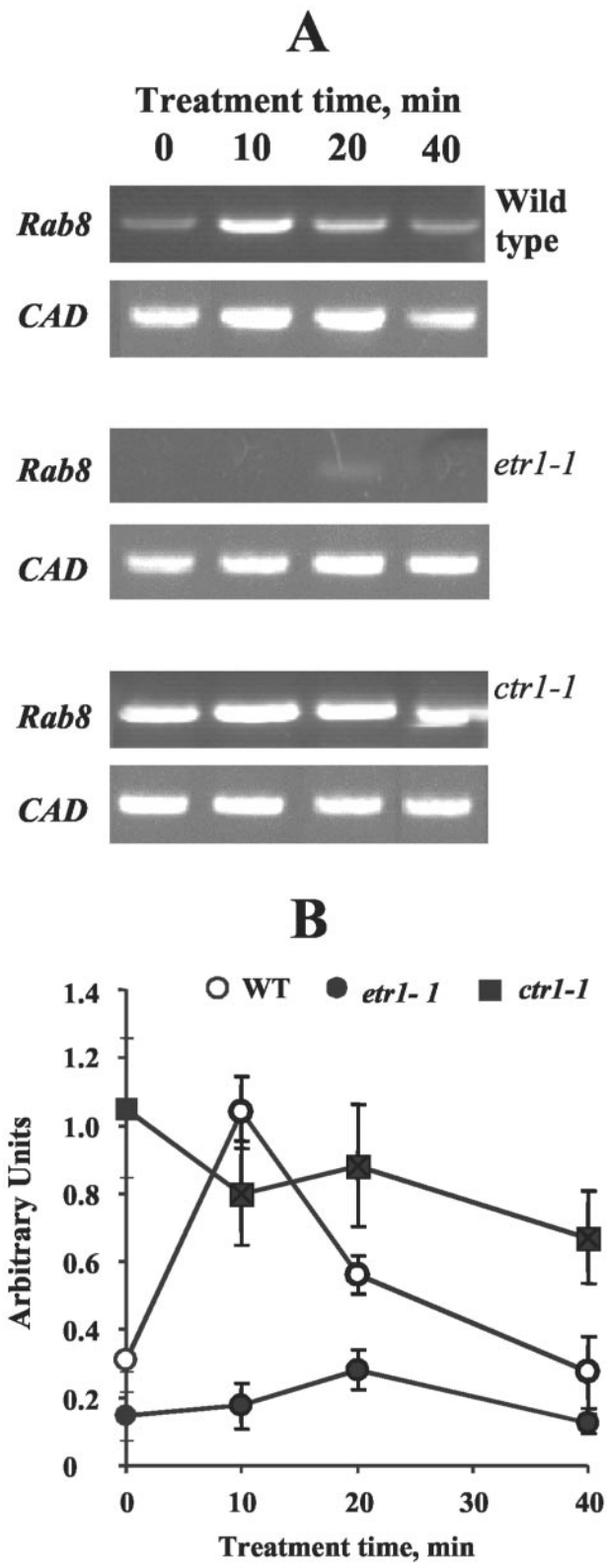

Figure 8. Rab8 expression in Arabidopsis wild-type and in ethylene signaling mutant plants. A, Wild-type Arabidopsis and the mutants *etr1-1* and *ctr1-1* were treated with ethylene for 0, 10, 20, and 40 min, and RNA was isolated from each of three Arabidopsis plants per time point. First strand cDNA was constructed from each plant. *Rab8* transcripts were detected using specific oligonucleotide primers from 1 μg of first strand cDNA. Control amplifications were carried out using oligonucleotide primers to *CAD*. Amplifications from each cDNA from each plant were repeated three times, representative results of which are illustrated. B, Monomeric G protein expression (of this and eight further gels) was quantified and equilibrated relative to the constitutive control, *CAD* levels in samples isolated from the same plant. Monomeric G protein expression is expressed in terms of "absolute" levels relative to *CAD*. Results are given as mean (n = 9) ± SE.

showed an even more severe phenotype than *ctr1-1*, implying that not all components of the ethylene signal transduction pathway are mediated via CTR1.

The relationship between the activation of monomeric G proteins and MAPKs in response to ethylene is unclear, although the pattern and timing of the two components is suggestive. Moreover, in *etr1-1*, overall protein phosphorylation and MAPK activation are down-regulated, whereas in *ctr1-1* these components are up-regulated (Novikova et al., 1999, 2000; Smith et al., 1999). In animal systems, the Ras group of monomeric G proteins appear to be most important in controlling MAPK cascades, and this group is absent in plants, at least in Arabidopsis (Arabidopsis Genome Initiative, 2000), and two-hybrid studies on CTR1 indicated a direct link between this protein and ETR1 (Clark et al., 1998). Li et al. (2001) have suggested that in plants, the role of Ras falls to Rop monomeric G proteins (Rho group). However, it should be noted that in animals, Rho and Rab proteins may have coordinate effects on development (Imamura et al., 1998), and a Rab-interacting protein (Rab8ip) is a Ser/Thr protein kinase (Takai et al., 2001). Furthermore, it is now well established that monomeric G proteins themselves form cascades (Van Aelst and D'Souza-Schorey, 1997; Campbell et al., 1998; Bishop and Hall, 2000), which may also account for the apparently large number that are activated by ethylene.

Whether or not the two types of component are linked, the possibility remains that they are involved in transduction chain(s) other than that controlled by CTR1 as we have argued elsewhere (Hall et al., 2001). This raises two questions. First, why have no sensitivity mutants for these components been obtained? It seems likely that this is due to functional redundancy, a common feature in animal signaling systems (Reuther and Der, 2000) and now shown for various components of the established ethylene transduction pathway (Hua and Meyerowitz, 1998; Tieman et al., 2000). It may be significant that the two monomeric G protein genes, transcription of which is shown here to be up-regulated by ethylene, code for proteins that are almost identical (approximately 93% homology). Equally, if it were not for the possibility of functional redundancy, it would be expected that sensitivity mutants would exist for the MAP2K(s) and MAPK(s) of the CTR1 cascade, but none have appeared so far, although it should be noted that whereas CTR1 and

Table III. *Primer sequences used in RT-PCR analysis of monomeric GTP-binding protein expression*

Designations	Sequence	Fragment Size and Intron No.
5′ Rab8	5′-GGACTAGTCCATGGCTGCTCCTCCTGCTAG-3′	584-bp cDNA, 1,729-bp genomic DNA, two introns
3′ Rab8	5′-CCGCTCGAGGTTTGCGGCTCAGCTCGTGC-3′	
5′ Ara3	5′-CCGCTCGAGCTTGCAGGCTCTGCCCTCGAG-3′	569-bp cDNA/ genomic DNA, no introns
3′ Ara3	5′-CTGATCGTTGCAGGCTCTGC-3′	
5′ Ara2/4	5′-CAAAGCTCAGATTTGGGATAC-3′	450-bp cDNA, 1,357-bp genomic DNA, one intron
3′ Ara2	5′-GTTAGAGCAGCAACCCATTC-3′	
5′ Ara2/4	5′-TTACCTCGAACAGCAAGAGAATG-3′	498-bp cDNA, 814-bp genomic DNA, one intron
3′ Ara4		
5′ Ara5	5′-TCGAAAACCATGAATCCTGAC-3′	631-bp cDNA, 1,443-bp genomic DNA, seven introns
3′ Ara5	5′-GAAGTTGCTTATTCCCAGCTGG-3′	
5′ Rac1, 5′ Rop4	5′-GTTYATAAAGTGTGTCACCG-3′	418-bp cDNA/ genomic DNA, no introns
3′ Rac4/Rop2/4	5′-CCTCTCCCTGGTTTGTAGTAATAGGCA-3′	
5′ Rac2/Rop5	5′-GCATCAAGGTTCATAAAGTGCGTC-3′	495-bp cDNA, no introns
3′ Rac1/2	5′-CKCCYTCACGTTCTCTTGTG-3′	

its two close homologs are Ser/Thr kinases, they only share about 60% homology with the Raf group and may not be true MAP3Ks.

The second question relates to whether the chain we propose acts wholly independently of the CTR1 chain but with opposite effects and is separately receptor-controlled or whether control is exercised via CTR1. With the present evidence, either is possible (or both). The fact that in *etr1-1*, where the receptor is locked into its active form and CTR1, therefore, is also active, the activities of both MAPK and monomeric G proteins are constitutively down-regulated can be taken to mean either that the receptor represses these activities directly or that CTR1 achieves it indirectly. In the latter connection, it is perhaps significant that MAPKs may inactivate monomeric G proteins through phosphorylation of guanine nucleotide exchange factors (GEP, which promote the exchange of GDP for GTP). The fact that in *ctr1-1* both MAPK and some monomeric G protein activities are constitutively up-regulated would also argue for this possibility. On the other hand, monomeric G proteins are normally directly receptor activated (albeit in a signaling complex), as are GEPs (Boguski and McCormick, 1993; Simon et al., 1993), and the rapidity of the activation in both peas and Arabidopsis argues for such a scenario. Whatever the answer, it is clear that CTR1 has a key role. It is notable that the effects of the *ctr1-1* mutation—up-regulation of protein phosphorylation (Smith et al., 1999) and MAPK activity (Novikova et al., 2000) and of both monomeric G protein activities and gene transcription shown here are strongly reminiscent of the effects seen in animals, where Raf-type MAP3Ks are mutated and become oncogenic (Heidecker et al., 1992). However, although in *ctr1-1* the lesion results in a loss of enzyme activity or the mutation is null (Kieber et al., 1993), in animals the oncogenic effects are due to constitutive activation of the enzyme.

Clearly, there is a need to define all the monomeric G proteins and MAPK(s) activated by ethylene and via transformation to attempt to define their role(s), work that is ongoing in our laboratory.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis plants (ecotype Columbia, wild type and mutants) were grown either in a greenhouse or in a controlled environment growth cabinet (daylength of 16 h, 20°C) in trays filled with Levington's compost and watered daily. Plants were collected at the early flowering stage (6 weeks old).

Rosettes minus roots (approximately 10 g fresh weight) were placed in sealed 1-L Kilner jars lined with moist filter paper to which 1 μL L^{-1} ethylene was applied for indicated time periods in the light at room temperature. MCP was applied at a concentration of 100 nL L^{-1} for 2 h before ethylene treatment. After treatment, the rosettes were used immediately for protein isolation or frozen in liquid nitrogen and stored at −70°C for RNA isolation.

Isolation of Membrane-Enriched Fractions

All procedures were carried out at 4°C. The rosettes were homogenized in freshly prepared buffer A (1:1.5 [w/v]), which contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mM diethyldithiocarbamic acid sodium salt, 5 mM ascorbic acid, 3.6 mM L-Cys, and 250 mM Suc. Polyvinylpolypyrrolidone was added to the buffer in a ratio of 1:10 (w/w) of plant tissue. The homogenate was filtered through 200-μm nylon mesh and the filtrate centrifuged at 12,000*g* for 20 min. The pellet was discarded and the supernatant centrifuged at 50,000*g* for 1 h. The pellet was discarded, and the supernatant was centrifuged at 130,000*g* for 3 h. The supernatant was discarded, and the pellet was resuspended in the same buffer supplemented with 20% (w/v) glycerol, divided into aliquots, frozen in liquid nitrogen, and stored at −70°C prior to protein solubilization.

Solubilization of Membrane Proteins

Resuspended membrane-enriched fractions were mixed (1:5 [v/v]) with buffer B containing 25 mM Na-HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride supplemented with KCl to give a final concentration of 100 mM and stirred for 30 min. The suspension was centrifuged at 130,000*g* for 2 h, and the supernatant was discarded because we have demonstrated previously that there was no specific ethylene-regulated GTP binding in this fraction (Novikova et al., 1997, 1999). The pellet was resuspended in buffer B but containing 750 mM KCl. After stirring for 30 min, the suspension was centrifuged at 130,000*g* for 1 h. The supernatant was collected and dialyzed overnight against 50 to 100 volumes of a buffer containing 25 mM Na-HEPES (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, and 2 mM EDTA. The pellet was resuspended in buffer B but containing 1% (w/v) Triton X-100. After stirring for 30 min, the suspension was centrifuged at 130,000*g* for 1 h and the detergent-solubilized fraction retained and dialyzed overnight against 50 to 100 volumes of 25 mM Na-HEPES (pH 7.5), 10 mM $MgCl_2$, 150 mM NaCl, 2 mM EDTA, and 0.05% (w/v) Triton X-100. The final pellet was then discarded. Protein content was

measured with BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL) according to the manufacturer's instructions.

Affinity Labeling with [α-^{32}P]GTP

Affinity labeling of GTP-binding proteins was carried out according to the method of Löw et al. (1992), using [α-^{32}P]GTP (specific activity 110 TBq mmol^{-1}; Amersham Pharmacia BioScience, Little Chalfont, UK). Reaction mixtures (25–50 μL), which included 25 to 50 μg of membrane protein extracted with either 750 mM KCl or 1% (w/v) Triton X-100 and 74 to 148 kBq [α-^{32}P]GTP, were incubated at 37°C for 10 min. $NaIO_4$ was then added to a final concentration of 4 mM and oxidation allowed to proceed for 1 min at 37°C. This was followed by reduction using $NaCNBH_3$ at a final concentration of 80 mM for 1 min at 37°C. Further reduction was then accomplished by the addition of $NaBH_4$ to a final concentration of 100 mM and incubation for 1.5 h at 0°C. Oxidizing and reducing agents were freshly prepared and kept at 0°C before use. The specificity of binding was assessed by using a 100-fold excess of unlabeled GTP. After labeling, the proteins were precipitated with 80% (v/v) acetone at −20°C and pelleted by centrifugation. The pellets were washed twice with 80% (v/v) acetone. For electrophoretic separation, proteins were dissolved either in sample buffer for SDS-PAGE (Laemmli, 1970) or sample buffer for two-dimensional electrophoresis (7.5 M urea, 2 M thiourea, 1% [w/v] Triton X-100, 4% [w/v] CHAPS, 20 mM DTT, and 0.2% [v/v] Pharmalyte [pH 3–10]; Amersham Pharmacia BioScience) to achieve a protein concentration of 2 mg mL^{-1}.

Electrophoresis

Labeled proteins were resolved using SDS-PAGE according to Laemmli (1970) or two-dimensional electrophoresis. Bio-Rad Mini-PROTEAN II and Mini Two-Dimensional Electrophoresis Cells were used. First dimension separation was carried out in 4% (w/v) polyacrylamide rods containing 9.2 M urea, 1% (w/v) Nonidet P-40, and 2% (v/v) Pharmalyte (pH 4.0–6.5; Amersham Pharmacia BioScience). Twenty micromolar NaOH was used as catholyte and 10 mM H_3PO_4 as anolyte. On the top of the rods, 5 μL of sample buffer was laid. The rods were prefocused as follows: 10 min at 200 V, 15 min at 300 V, and 15 min at 400 V. Then, the catholyte and anolyte solutions were discarded and all the liquid from the rods was removed and replaced with fresh catholyte. Protein samples (20–50 μg) were loaded on the top of the rods and covered with overlay buffer containing 3.5 M urea, 0.5% (w/v) Triton X-100, and 0.5% (v/v) Pharmalyte 3–10. The running conditions were as follows: 15 min at 500 V and 4 h at 750 V. After isoelectrofocusing, the gels were carefully removed from glass capillaries and equilibrated for 20 min in SDS-PAGE sample buffer. The rods were then placed on the top of 12.5% (w/v) PAGE 1 mm thick and subjected to electrophoresis at 200 V. After electrophoresis, the gels were fixed, stained, dried, and subjected to autoradiography.

Extraction of Total RNA and Poly(A$^+$)

Frozen rosettes were easily detached, and RNA was extracted only from mature fully expanded leaves; typically, four leaves were taken from each plant. Leaf samples were ground down in a liquid nitrogen-cooled pestle and mortar. The ground material was transferred into a measuring cylinder and an equal volume of RNA extraction buffer (8 M guanidine-HCl, 20 mM EDTA, and 20 mM MES [pH 7]) was added and vortexed. Samples were transferred to 50-mL Oakridge tubes, and proteins were extracted with the addition of an equal volume of phenol:chloroform (1:1 [v/v] equilibrated with 100 mM Tris [pH 7.2]), followed by centrifugation (3,500*g* for 5 min) and removal of the upper aqueous layer to 50-mL Corex tubes. RNA was precipitated by the addition of 1/20th volumes of 1 M acetic acid and 0.7 volumes of absolute ethanol and incubation on ice for 30 min. After centrifugation, the pellet was washed sequentially in 3 M sodium acetate and 70% (v/v) ethanol. The pellet was air dried and resuspended in RNAse-free water.

Poly(A$^+$) was extracted from 500 μg of total RNA using the Poly(A$^+$) Tract mRNA isolation system (Promega, Madison, WI) following the manufacturer's instructions. The final poly(A$^+$) concentration was estimated by spectroscopy, and typical yields were approximately 5 μg. The quality of the poly(A$^+$) as a template for DNA synthesis was assessed by measuring the incorporation of radioactive [α-^{32}P]dCTP (specific activity 110 TBq mmol^{-1}, Amersham Pharmacia BioScience; +10 mM dATP, dGTP, and dTTP, Promega) into cDNA using a Superscript II kit (Invitrogen, Paisley, UK) as recommended by the manufacturer. All samples used for transcriptional analysis exhibited >50% incorporation of the radiolabel.

Amplification of mRNA Transcripts for Specific Monomeric G Proteins

First strand cDNA was synthesized from 5 μg of poly(A$^+$) using a Superscript II (Invitrogen) following the manufacturer's instructions. A series of oligonucleotide primers were designed that in combination would amplify mRNAs for specific monomeric G proteins. Due to the extensive conservation in monomeric G protein nucleotide sequence, individual primers would bind to a range of monomeric G protein genes. These are referred to in the oligonucleotide designations, which precede sequences given in Table III. Fragment sizes when amplifying from either cDNA or genomic DNA and the number of introns, which are spanned, are indicated.

CAD transcript accumulation was used as an internal control (Somers et al., 1995) in each RNA sample (5′Cad 5′-GGCAGGGAAGCTTTAGGGG-3′ and 3′Cad 5′-AGTTAGCCACGTCGATCACG-3′; 480-bp cDNA, 675-bp genomic DNA, and one intron). Approximately 1 μg of cDNA was used as template in each PCR. The PCR amplification cycle that was used was invariably 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min (30 cycles). Before first use, each primer combination was tested with genomic DNA to confirm that only the expected fragment size was obtained. The entire amplified sample was loaded on to the gel to give the results presented. Preliminary analyses were carried out where samples from PCR reactions were taken after 10, 20, 22, 25, 27, and 30 cycles, and the DNA bands visualized on an agarose gel. This established that in each case, amplification from the target sequences was linear (data not shown).

Quantification of GTP Binding on Autoradiographs and Ethidium Bromide-Stained DNA on Agarose Gels

[α-^{32}P]GTP binding to monomeric G proteins as detected on autoradiographs was quantified using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA), which qualified pixel intensity over a designated, in this case, circled area. To compare binding between gels, all distinct [α-^{32}P]GTP-binding components on gels were annotated, circled, and scanned for pixel intensity. All ethidium bromide-stained RT-PCR DNA bands were scanned using a Typhoon 8600 Imager (Molecular Dynamics) in fluorescence mode and quantified using ImageQuant Software.

Monomeric G protein lineups were generated using ClustalX (Thompson et al., 1997) from sequence held at the GenBank database and non-rooted phylogenetic trees using Phylo-Win Version 1.2 (Galtier et al., 1996). Estimations of M_r and pI were made using Compute pI/Mw (http://www.expasy.ch/tools/pi tool.html; Bjellqvist et al., 1993). Screens for membrane-associated regions were made using T-MAP (Persson and Argos, 1994) at the Biology WorkBench (http://biowb.sdsc.edu/CGI/BW.cgi).

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