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INTERACTIONS BETWEEN NITRIC OXIDE AND ETHYLENE IN MONOMERIC G-PROTEIN ACTIVATION IN RELATION TO FOOD SPOILAGE

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Summary: Climate change is likely to increase crop stress with negative impacts on yield and quality. Therefore, there is a need to develop our understanding of the key events which govern plant tolerance to stress. Intense research has identified key signalling cascades regulating stress tolerance and it is notable that many are dependent on the production of volatile signals or signals which have volatile derivatives. Ethylene (ET) has long been recognized as an important regulator of development, stress responses, senescence and food spoilage. Our work has focused on the gaseous signal nitric oxide (NO) and how it interacts with established stress signalling pathways and in particular, those regulated by ET. Using laser photoacoustic detection (LPAD) we have established that NO production overlaps with that of ethylene during plant responses to disease. To examine the interaction of NO and ET signalling we focused on the activation of monomeric GTP binding proteins (MGBP) which we have previously shown to be components of ET signalling cascades. MGBP activation following application of sodium nitroprusside (SNP) an NO^+ donor or ET was compared in wild type Col-0 *Arabidopsis* plants. Using a proteomic approach and 2D-electrophoresis (2DE) a series of GTP binding proteins which were activated by both ethylene and SNP were detected and some that exhibited specific activation patterns in response to both signals. These observations underline the close relationship between ET and NO signalling cascades and possibility of NO being assessed as part of a plant produce stress volatilome.

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INTRODUCTION

In recent years a significant effort has been directed towards studying methodologies, e.g. packaging/artificial atmospheres/temperature profiles, in order to prolong product life and to combat the effect of spoilage microorganisms, on quality and associated safety concerns, with considerable success. However, whatever the strategy that is employed, losses in storage, handling, transport, in retailers and by customers remain significant. Although, varying greatly with country of origin and type of produce, these losses can be as much as 40% and are rarely less than 10% overall. Post-harvest losses in horticultural fruit and vegetable crops are mainly related to handling, from harvest to retail. It is imperative therefore that such losses at whatever stage and from whatever cause – e.g. environmental and mechanical stresses to the crop before shipping causing accelerated ageing, the presence of spoilage organisms, etc. – are recognized at the earliest possible stage and material unfit for purpose discarded.

Plant scientists have recognised that detection of plant stress volatiles have the potential to represent a non-intrusive 'early warning' indicator of problems in either growing crops or in the harvested plant products. Volatile compounds forming the stress "volatilome" are produced in large amounts and production is not only at the site of injury, for example a wound or pathogenic challenge, but in other regions of the plant (Bicchi & Maffei, 2012). The predominant volatiles produced are phospholipid-derived green leaf volatiles (GLV) such as hexanal, hexane, and hexane acetate and a wide range of terpenoids (Matsui, 2006). Additionally, the production of the wound- and senescence-responsive volatile, ET (Johnson & Ecker, 1998) is also an indicator of plant produce stress.

In the last decade of the 20th Century, the role of the gaseous signal NO in both plant development and also responses to biotic and abiotic stress came to be recognised. NO has been implicated in defence against *Pseudomonas syringae* pathogens (Clarke et al., 2000, Delledonne et al., 1998, Mur et al., 2005); in barley infected with powdery mildew and downy mildew on pearl millet (Prats et al., 2005, Manjunatha et al., 2009) or *Botrytis cinerea* challenged *Arabdiopsis* (Lloyd et al., 2011). These observations suggest that NO could be similarly used as another important indicator of stress in the plant volatilome.

However, NO has often been reported to have a suppressive effect on ET production and signalling. Leshem & Pinchasov, (2000) used laser photoacoustic detection to measure both NO and ET in ripening avocados and strawberry and noted that, on ripening, NO levels were reduced as ET increased. A mechanistic understanding of this interaction was provided by (Lindermayr et al., 2006, Lindermayr et al., 2010)). The Yang (methylmethionine) cycle produces S-adenosylmethionine (AdoMet) which is the methyl donor linked to the production of a range of metabolites including ET and also polyamines (Roje, 2006). Lindermayr et al., (2006) reported that NO-dependent *S*-nitrosylation of a key cysteine (Cys-114) within the active site of a methionine adenosyltransferase (MAT1; At1g02500) suppressed MAT1 enzymatic activity and also ET production. Against such results our group has reported several examples

where there is a simultaneous generation of both NO and ET (Mur et al., 2008a, 2009, 2012); for example during the elicitation of a hypersenstive response by the bacterial pathogenic strain *Pseudomonas syringae* in *Arabidopsis* (Fig. 1). Further, we have reported that the NO^+ donor – sodium nitroprusside (SNP) – following infiltration into tobacco leaves elicited the production of ethylene (Mur et al., 2005, 2008b). As SNP could induce ACC synthase expression (ACS), this seems to be one mechanism through which NO could boost ET production (Mur et al., 2008). Similarly, the expression of mammalian *NOS* in transgenic tobacco increased ACC oxidase (the final enzyme in ET biosynthesis) and *ethylene-responsive element binding protein* (*EREBP*) expression (Chun et al., 2012).

Our group has previously established that MGBPs are components of the ET signalling cascade (Fig. 2) and now assessed if they play a role in NO signalling. Using a proteomic approach, commonly activated MGBPs were detected as well as MGBPs with distinctive activation patterns.

MATERIAL AND METHODS

Plant Material and Treatments

Arabidopsis wild-type plants (ecotype Columbia, Col-0) were cultivated on Levington Universal compost in trays with 24 compartment inserts. Plants were maintained in Conviron (Controlled Environments Ltd, UK) growth rooms at 24°C with a light intensity of 110 µmol/m/s and an 8 h photoperiod for 5 weeks.

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} ET was applied for indicated time periods in the light at room temperature. For NO treatments, plants were sprayed with 100 µM SNP or 100 µM "spent SNP" (where NO has been driven off from a SNP by being left in daylight for 2 days); and sealed 1-L Kilner jars for

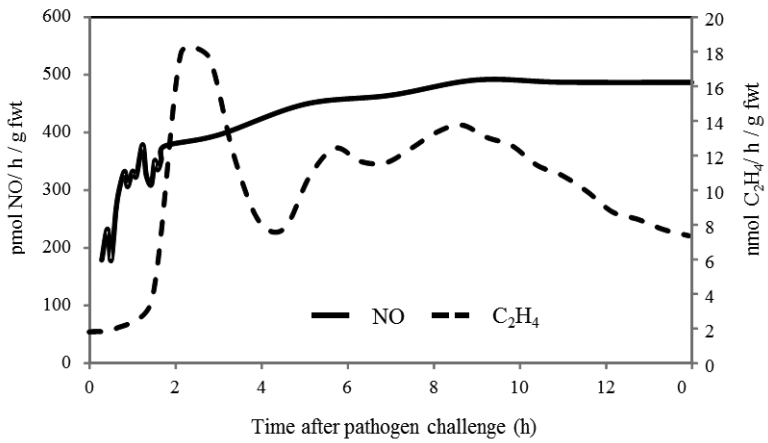

Figure 1. Pathogen-elicited nitric oxide and ethylene production. Nitric oxide (NO) and ethylene production was determined using laser photoacoustic following inoculation with *Pseudomonas syringae* pv. *tomato avrRpm1*. Results are given as mean pmol (for NO) or nmol (for ethylene) per g fresh weight (g fwt).

a given time period. 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 MgCl2, 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 sucrose. 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 solubilisation.

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 and 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 Labelling with [α-^{32}P]GTP

Affinity labelling 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 unlabelled GTP. After labelling, 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 (2DE) (6 M urea, 1.5 M thiourea, 3% [w/v] CHAPS, 66 mM DTT and 0.2% [v/v] Bio-Lytes [pH 3–10]; Bio-Rad) to achieve a protein concentration of 2 mg mL^{-1}.

Electrophoresis

Labelled proteins were resolved using SDS-PAGE according to Laemmli (1970) or 2DE. Bio-Rad Mini-PROTEAN III and Bio-Rad Protean IEF Cell were used. For the first dimension, labelled proteins were dissolved in rehydration buffer (130 µL) contained 6 M urea, 1.5 M thiourea, 3% CHAPS, 66 mM DTT, 0.2% Bio-Lytes (pH 3–10, Bio-Rad), and traces of bromophenol blue. Protein samples (50–100 µg) were loaded on IPG strips (7 cm, pH 4–7; ReadyStrips, Bio-Rad) for passive rehydration at 20^0C for 12 h. The running conditions were as follows: 200 V constant voltage for 15 min, 500 V constant voltage for 15 min, linear increase up to 1000 V for 30 min, slow increase up to 5000 V for 30 min, and 5000 V constant voltage until a total of 10 000 V h was reached. After IEF, the strips were equilibrated for 15 min with the buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 10 mg/ml DTT followed by equilibration for 15 min with the same buffer, which contained 25 mg/ml iodoacetamide instead of DTT. The strips were then placed on the top of 12.5% polyacrylamide mini gels (0.75 mm thick) and subjected to SDS-PAGE at 200 V. After electrophoresis, the gels were fixed, stained, dried, and subjected to autoradiography on a Kodak Biomax MR film.

Data analysis

Imaged 2DE gels were analysed using Progenesis PG220 v.2006 (previously Phoretix 2D Evolution v.2005). Analysis was performed on autoradiographs from a minimum of 3 biological replicates. Normalised spot volumes on the autoradiographs were achieved using total spot volume multiplied by total area and were also used to determine any increase or decrease in protein abundance between comparisons (with significance set at +/- 2-fold change).

RESULTS AND DISCUSSION

The elucidation of components of the ET signal transduction pathway have been effectively characterised mainly through studies on *Arabidopsis* mutants (Hall et al., 2001) (Fig. 2). These have shown ET perception to be based on at least five partially functionally redundant receptors which appear to be negative regulators (ETR1, ETR2, ERS1, ERS2, and EIN4) so that they are active in the absence of ethylene and inactive in its presence (Hua & Meyerowitz, 1998). The receptors acting through a protein (CTR1) having homology with mammalian Raf-type mitogen-activated protein kinase kinase kinases (MAPKKK). In both animals and yeasts, MGBPs lie upstream

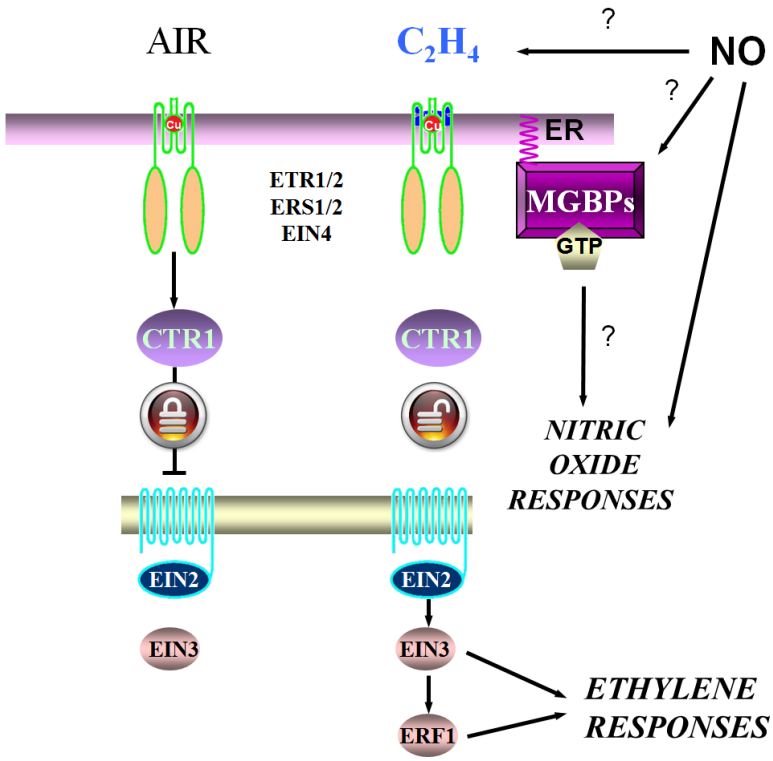

Figure 2. A simplified ethylene signalling cascade highlighting the role of monomeric GTP-binding proteins and the possible role of nitric oxide. Interrelationships between signal transduction components involving monomeric G-proteins (MGBPs) and mitogen-activated protein (MAP) kinase cascades.

of MAPKKKs (of both the Raf- and MEKK-types) which they may activate directly or indirectly through another protein kinase (Hall et al., 2001). Our extensive analyses have demonstrated that ET signalling is associated with differential activation of MGBPs that could be involved in linked receptor to MAPK signalling cascades and other ET-dependent outputs (Moshkov et al., 2003a, Moshkov et al., 2003b). In plants, the differential activation of MGBPs with NO has not been determined, however they are well established in mammals (Mitchell et al., 2013). Following on from each reports showing that NO activates the mitogen-responsive MGBP(s), p21/Ras (Lander et al., 1996) and many other events; for example smooth muscle cell proliferation, have been shown to be influenced by NO modulation of Ras and Rho MGBPs (Rikitake & Liao, 2005, Zuckerbraun et al., 2007, Mitchell et al., 2013). In this study, we sought to establish if MGBP activities could be influenced by an NO donor and compare activation patterns to that seen with ET. Ethylene can be efficiently applied as a gas but the highly reactive nature of NO results in a half-life of ~ 30 sec (Wink et al., 1996). By contrast we have observed a steady release of NO with the NO^+ donor-SNP over many hours; a kinetic pattern that was unique to all of the commercially

available NO donors that we tested which released their NO immediately on going into solution (Mur et al., 2013).

Thus, five week-old wild-type Col-0 *Arabidopsis* plants were either simply incubated in a Kilner jar, or incubated gassed with 1 µL L^{-1} ET, or sprayed with 100 mM donor SNP or 100 mM spent SNP. Samples were taken at 20 and 60 min and the membrane fractions containing the MGBPs were harvested as described in the methods.

To reveal MGBP activities, we employed *in vitro* [α-^{32}P]GTP binding assay, followed by a 2DE separation based on p*I* and molecular weight. The [α-^{32}P]GTP-bound MGBPs were visualised by autoradiography (Fig. 3). GTP binding was compared to each controls for ET (Kilner jar incubation) or for SNP (spent SNP in a Kilner jar) to identify constitutive GTP binding protein or proteins that were induced by non-ET/NO mediated effects.

Each spot of GTP binding was given a designation and each gel result was aligned and analysed as if protein abundance were being described, using Progenesis software (Fig. 3). These analyses led to the identification of ten spots of GTP-binding activity on the autoradiographs which were designated. The activity for each spot at 20 and 60 min was expressed as fold difference over the values at 0 min. The values for each spot, over time were displayed using a heat map and compared using hierarchical cluster analyses (HCA) (Fig. 4).

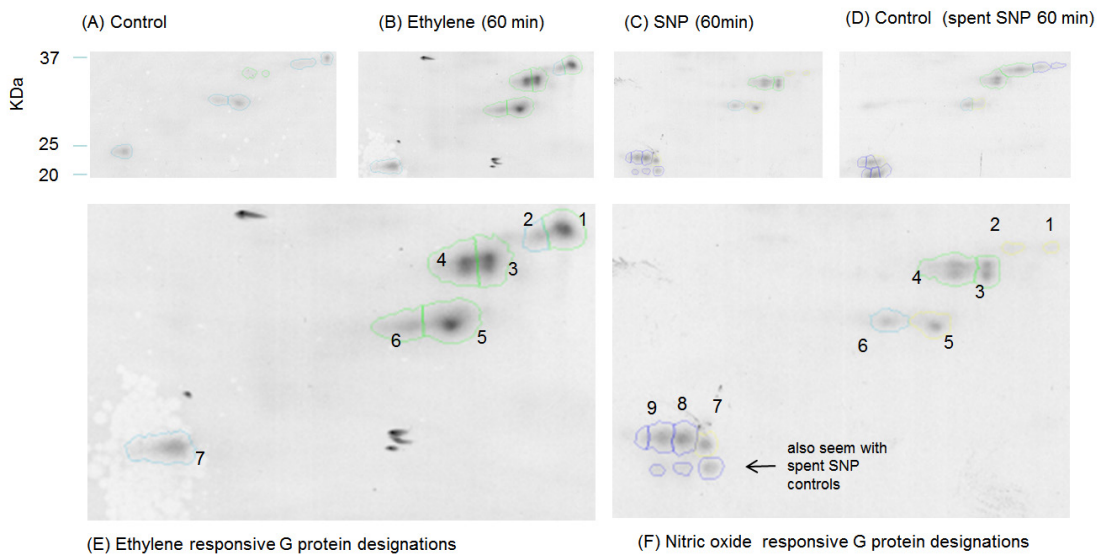

Figure 3. Proteomic assessments of ethylene- and NO-activated monomeric GTP-binding proteins. Autoradiographs of [α-^{32}P]GTP binding to *Arabidopsis* proteins separated by a two-dimensional gel electrophoresis based on p*I* and molecular weight. [α-^{32}P]GTP binding in proteins from *Arabidopsis* plants after 1-h incubation in (A) Kilner jars ;(B) Kilner jars with 1 µL L^{-1} ethylene; (C) sprayed with 100 mM sodium nitroprusside (SNP) in Kilner jars or (D) sprayed with 100 mM "spent" SNP (where NO had been driven off from the solution following 2 day illumination with light) in Kilner jars. (E) and (F) representations, respectively, of (B) and (C) with spot designations.

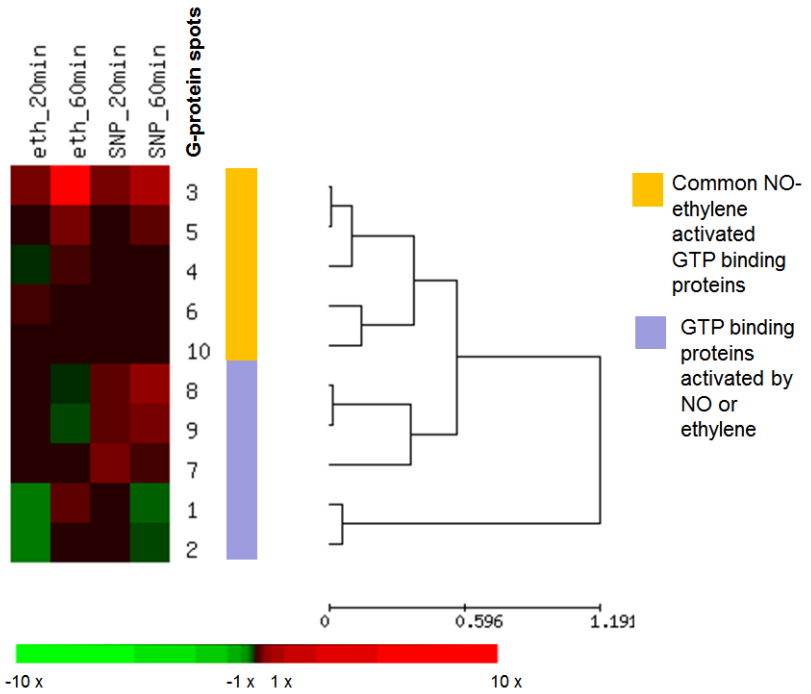

Figure 4. Comparison of ethylene- and NO-activated monomeric GTP-binding proteins. [α-^{32}P]GTP binding in discrete *Arabidopsis* proteins was quantified from autoradiographs using Phoretix software. The binding of individual spots was expressed as a fold difference over relevant controls; i.e. plants in Kilner jar (without ethylene) for ethylene treatments and plants sprayed with spent SNP when NO effects were assessed. Fold differences were $\log_2$ transformed. Changes in GTP binding illustrated using a heat map and grouped by Hierarchical Cluster Analysis. GTP-binding proteins which are activated by both ethylene and NO or by individual signals are indicated.

The HCA broadly separated the GTP-binding proteins into those which appeared to be activated by both ET and SNP/NO and those which appeared to have distinctive activation patterns. Considering the commonly activated MGBPs (3, 4, 5, 6, 10) the differences between ET and NO appeared one of extent of binding rather than pattern (compare Fig. 3 B with 3C). This may reflect differences in the relative concentrations of the gaseous signals which were difficult to assess in the Kilner jars. Alternatively, it may be that NO acts on these MGBPs through the initiation of ET production (Mur et al., 2008); thus reducing the strength of induction. However, the more distinctive activation pattern seen for ET (spot 1, 2) or SNP (8, 9) would suggest unique signal-specific impacts on MGBP-mediated signalling. These GTP-binding activities are currently being targeted by our groups.

Within the context of the food spoilage assessment, the commonality of certain MGBP signalling nodes suggests that NO and ET can be considered as good detection targets. However, the specificity of certain MGBP activation patterns argues for different roles that could mean that the production of different gases could be used as a diagnostic for different stresses. We are currently testing these hypotheses.

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