

2014

Methanol and Ethanol Modulate Responses to Danger- and Microbe- Associated Molecular Patterns in Tomato, Tobacco and Arabidopsis

Claire Teresa Hann

University of South Carolina - Columbia

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METHANOL AND ETHANOL MODULATE RESPONSES TO DANGER- AND MICROBE-
ASSOCIATED MOLECULAR PATTERNS IN TOMATO, TOBACCO AND ARABIDOPSIS

by

Claire T. Hann

Bachelor of Science
Presbyterian College, 2010

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2014

Accepted by:

Johannes W. Stratmann, Director of Thesis

Deanna Smith, Reader

Zheng Qing Fu, Reader

Lacy Ford, Vice Provost and Dean of Graduate Studies

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DEDICATION

I dedicate this thesis to my grandfather Luigi Punzo, who loved growing tomatoes, and who gave me my first memories of life in the garden.

ACKNOWLEDGEMENTS

I would first like to acknowledge Dr. Sarah Refi-Hind for introducing me to techniques in molecular biology. I would then like to thank Dr. Johannes Stratmann for his continued optimism and support in the face of doubt in both myself, and the results I obtained. It has been a privilege to work with both scientists, for through their expertise and patience they gave me the tools necessary to perform my investigations. I would also like to thank Ph. D. candidate Carlton J. Bequette in the Stratmann Lab for being an excellent role model of everyday professionalism and a friend in the lab. He was a constant source of help and support, always kind, and always cooperative in my endeavors. Dr. Erika Balogh piqued my curiosity in plant science, and gave me the confidence to consider being a member of the scientific community. She has been an indispensable companion. Also, Dr. Soumitra Ghoshroy and Dr. Mihaly Czako were very helpful in teaching me skills related to various aspects of my research at USC, and supported me in my application process to the university. They were always available when I needed advice. I would like to give a special thanks to Dr. John Mervin Herr for reminding me that science is a goal in and of itself, that it is worth struggling for, and that one should continue relentlessly until one has achieved it. His philosophy has helped solidify the way I view study, and for that I will always be grateful. This work was supported by National Science Foundation grant IOS-0745545 to J.W.S.

ABSTRACT

Methanol is a byproduct of cell wall modification. It is released through the action of pectin methylesterases (PMEs), which demethylate cell wall pectins. Plant PMEs play not only a role in developmental processes but also in responses to herbivory and infection by fungal or microbial pathogens, resulting in increased methanol release. To break down the cell wall barrier, pathogens employ their own PMEs. Interestingly, the infection process also regulates the expression of certain plant PMEs. Methanol is toxic to a number of herbivores and reduces their fitness. At high concentrations, it upregulates signaling and defense genes. However, molecular mechanisms that explain how methanol affects plant defenses are poorly understood. Here we show that methanol alone has weak effects on defense signaling, however it profoundly alters signaling responses to danger- and microbe-associated molecular patterns (DAMPs, MAMPs) such as the alarm hormone systemin, the bacterial flagellum-derived flg22 peptide, and the fungal cell wall-derived oligosaccharide chitosan. Methanol application shifts DAMP/MAMP-induced MAP kinase (MAPK) activity in tobacco and tomato cell cultures, as well as *Arabidopsis* seedlings. It also shifts a flg22-induced ROS burst in tomato leaf tissue. We propose that the cell wall breakdown product methanol is perceived by plant cells as a DAMP-like alarm signal that alters defense responses to other DAMPs and MAMPs.

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LIST OF ABBREVIATIONS

CHT	Chitosan
CWDE	Cell wall degrading enzyme
FLG	Flg22
DAMP	Danger-associated molecular pattern
HG.....	Homogalacturonan
MAMP	Microbe-associated molecular pattern
MAPK6.....	Mitogen activated protein kinase 6
OG	Oligogalacturonide
PGA.....	Polygalacturonic acid
PI II.....	Proteinase Inhibitor II
PME	Pectin methylesterase
PMEI	Pectin methylesterase inhibitor
RIDA.....	Radial Immunodiffusion Assay
SYS.....	Systemin

CHAPTER 1: INTRODUCTION

As sessile organisms, plants have evolved sophisticated ways of adapting to stressors in their environment. These mechanisms are responsible for allowing plant organisms to both perceive and respond to threats in ways specific to the stressor. A principal mode of perception in plants involves the activity of receptors, which by binding a ligand can initiate a signaling module that allows for protective transcriptional responses. Our work focuses on the MAP kinase cascade, which transduces environmental and developmental cues into adaptive responses.

MAP kinases regulate a variety of processes throughout the life of the plant, including developmental systems, stress and hormonal responses, and innate immunity. They do so by transiently and reversibly phosphorylating members in the kinase cascade in a sequential manner to relay a signal (29). A MAP triple kinase (MAP3K) is phosphorylated when a cell surface receptor, often a receptor-like kinase (RLK), binds a ligand. The MAP3K then phosphorylates a MAP kinase kinase (MAP2K), which in turn phosphorylates a MAP kinase (MAPK) (2). MAPK substrates can include proteins in the nucleus or the cytoplasm, such as other kinases, enzymes, or transcription factors. At the end of the cascade, the phosphorylation substrates of MAPKs are activated, aiding survival by causing a response to the ligand stimulus (25, 29).

Ligand stimuli are classified by their origins, which often predict the signaling

pathway necessary to effectively respond to the detected threat. Typically there is a high degree of specificity between ligands and receptors, but new evidence suggests that some receptors can be activated by more than one ligand to orchestrate a separate process (2). Although we are aware of many defense elicitors in plants, not all receptors responsible for their perception have been identified (25).

Ligands derived from biotic stressors are grouped into microbe associated molecular patterns (MAMPs), plant-derived danger associated molecular patterns (DAMPs), effector proteins and herbivore elicitors. MAMPs are highly conserved microbial compounds. The best-studied MAMP is flg22, the most active component of the bacterial flagellin protein (12). DAMPs are considered “self” danger signals, and include byproducts of cell wall degrading enzyme activity such as oligogalacturonides (OGs) (9), or plant peptides synthesized in response to pathogen infection (Peps) (16) and herbivory (systemin) (13). Herbivore elicitors are detected in the saliva of chewing insects, and have been shown to include a variety of chemicals such as fatty acid conjugates (3).

In *Arabidopsis thaliana*, when a cell surface receptor detects a MAMP from bacterial or fungal pathogens, MAPK6 is rapidly activated by phosphorylation and remains active for a short period of time (29). This protein is called MAPK1/2 in tomato, and SIPK in tobacco, but from now on we will refer to it as MAPK6. Its phosphorylation is considered an indication that perception has occurred, similar to a “readout” of perception. This phosphorylation usually lasts less than an hour, due to inactivation via dephosphorylation by MAP kinase phosphatases (MKPs) (25). In the meantime, MAPK6

phosphorylates transcription factors responsible for transcriptional changes that regulate the early response, especially the expression of pathogenesis related (PR) genes, generation of ROS, or the rate-limiting enzyme for ethylene biosynthesis, 1-Aminocyclopropane-1-Carboxylic Acid Synthase (18).

Responses to MAMPs also depend on other cellular mechanisms known to have their own regulatory activity in defense, in addition to imparting specificity to MAPK6 activity. This is especially the case when the cell senses the influx of apoplastic Ca^{2+} that occurs upon elicitor perception via an RLK (19). The presence of Ca^{2+} activates a calcium-sensitive signaling network. The timing and intensity of the influx is believed to regulate the behavior of multiple independent players in signal transduction, such as regulatory protein calmodulin, calcium-dependent protein kinases (CPKs) and calcineurin B-like interacting protein kinases (CBL-CIPKs) (11, 29). Their signaling networks have important roles in the activation of late defense genes, such as those involved in the production of salicylic acid and the accumulation of antimicrobial phytoalexins, but they also interact with the MAPK cascade (4). Therefore, a great amount of crosstalk occurs between multiple modes of signal transduction upon perception of a threat. This suggests that the activity of specific components of the network at particular times may have a profound effect on the output response generated.

Apart from receptors at the cell's surface, the structurally complex cell wall is the first line of defense against an invading pathogen (29). At the same time, the regulation of cell wall characteristics during the course of development is essential to proper

growth and organ formation. Growth must be adjusted in accordance with cell wall stability, which requires that the cytoplasm receive information from the cell wall (30, 31, 32). Also, the deposition of a secondary cell wall in specialized cell types can be a strategy for energy storage in some tissues, or for structural reinforcement of elongating structures. Although the nature of the signaling mechanisms employed during cell wall integrity surveillance is currently unclear, it is already well understood that this essential developmental function is closely connected to defense (20).

The intricate polysaccharide composition of the primary cell wall presents a variegated challenge for pathogens. Cell wall composition varies significantly between species, but generally cellulose makes up about 30% of the cell wall (32). Its tightly packed microfibril arrangement makes it difficult to penetrate, while providing the tensile strength necessary to handle increases in turgor pressure. Microfibrils are highly oriented in accordance with the mechanical requirements of the direction of growth (26).

Flexibility and stability of the wall is however dependent on other polysaccharides in the matrix, which are required for reinforcement of the microfibril arrangement and to prevent nascent microfibrils from overaggregating. Hemicelluloses, long polymers with short branches, crosslink microfibrils through hydrogen bonding. Both hemicellulose and cellulose also interact with pectin, which modulates the fluidity of the cell wall. The interaction of the three is important in cell wall integrity (32). However, the lack of severe growth defects in hemicellulose deficient *Arabidopsis*

mutants has suggested that the function of hemicellulose in the cell wall is partially redundant to that of pectins (7).

In dicots, pectins make up 30%-50% of the primary cell wall, while in monocots this number is closer to 10% (17). Pectin is made up of a group of both linear and highly branched polysaccharides covalently bonded together. Whether these are bonded end-to-end, or as side chains of each other, or in both configurations has not been clarified. Pectin components include the more linear homogalacturonans (HG) and xylogalacturonans (XGA), in addition to the more branched and complex rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). RG I is thought to have close interaction with cellulose through covalent bonding, while RG II has been shown to act as a crosslinker between HG polymers through boron substitutions. With the exception of RG I, which has an L-rhamnose and D-galacturonic acid backbone, these polysaccharides have backbones composed of alpha-1,4-linked D-galacturonic acid referred to as galacturonan (17, 32).

In order to manipulate the plant cell wall, both plants and pathogens express a variety of cell wall degrading enzymes (CWDEs) from the glycoside hydrolase, carbohydrate esterase, and polysaccharide lyase families (5, 11). The stabilizing effect of pectin's helical and branched structure makes its architecture the target of pectic enzymes from both organisms. The activity of multiple pectic enzymes can have profound effects on cell wall integrity due to the access one enzyme can create for another, their concerted efforts eventually changing the fluidity of the matrix (5, 11, 17, 29).

HG polymers are the most abundant galacturonans in plant cell walls, and compose much of the middle lamella. Although their length and linearity point to functions as possible backbones or sidechains to other pectins, their ability to self-adhere is also a component of their highly dynamic contribution to cell wall function (6, 31, 32). The D-galacturonic acid residues are methyl-esterified during synthesis in the Golgi, and demethylesterification of these residues *in muro* exposes negatively charged carboxyl groups to Ca^{2+} ions. Ca^{2+} then acts as a readily available crosslinker between HG polymers. This means that during development, the activity of plant pectin methylesterases (PMEs) can stiffen the cell wall, causing HG polymers to form a gel (6, 17). Fine-tuning the stiffness of the cell wall is important in phases of cell wall growth such as cell lengthening or expansion; PME's activity in that respect is known to be regulated by PME inhibitors (30).

At the same time, the activity of PME can increase cell wall fluidity through the action of other enzymes, such as the pectin hydrolase polygalacturonase. The removal of methyl groups by PME exposes the glycosidic bonds of HG to polygalacturonase and pectic lyases, which cleave the long polymer into fragments. Plants inhibit pathogen polygalacturonases with polygalacturonase inhibitor proteins (PGIPs) embedded in the cell wall (17, 28). PGIPs are known to slow the action of the pathogen enzyme so that it may produce longer oligogalacturonide fragments. Longer oligogalacturonides are more easily sensed by cell surface receptors, and can therefore act as DAMPs, but they must be demethylesterified to be active. PME is therefore required for the generation of OGs as DAMPs (17, 22).

Multiple genetic studies have shown that the expression of pathogen PME is a critical requirement for virulence (17, 24). At the same time, a higher degree of cell wall methylation has correlated with disease resistance in multiple plant species (17). Plant PME can also be induced during infection (25). Taking into consideration how critical the PME-HG interaction is to cell wall invasion, and how tightly the plant cell regulates cell wall integrity, it stands that the cell may have multiple mechanisms in place for sensing the activity of foreign PME.

One way to perceive this type of PME activity might be the detection of methanol. Methanol is emitted in large quantities by plants via the action of PME (21). The implication of PME in the bulk of methanol emission has been studied via both overexpression and mutation of the enzyme (8). Its volatility allows it to rapidly exit the leaf tissue via the stomata. The amount of methanol emitted is higher in young plants, and is reduced in mature leaves (30), which is consistent with PME's activity profile during growth. Recent improvements in technology have made it possible to measure plants' methanol emission in real time, which has led to the discovery that biotic stress events such as herbivory can increase the emission of methanol through PME activity (8, 15, 23, 24).

Microarray studies in *A. thaliana* have shown that exogenous application of 10% methanol causes the upregulation of about 500 genes, or 1.9% of the 25,000 represented genes. Amongst these, the most highly represented are components of cellular communication and signal transduction pathways, specifically kinases and protein phosphatases. A wall-associated kinase (WAK3) was upregulated 1.6 fold after

24 hrs, which suggests that plant cells might be able to sense methanol in the cell wall, or at least, that methanol may alter the cell wall's sensitivity to other ligands. In addition, methanol upregulated the expression of PR1 and its precursor, transcripts implicated in JA synthesis, e.g. oxophytodienoic acid (OPR1), genes involved in ethylene signaling such as the ethylene biosynthetic enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACO), and calcium signaling proteins (10).

The detection of methanol released from HG at an inopportune time may serve as an additional warning signal that cell wall integrity is threatened. We offer this explanation for the results we have obtained during the study of methanol's effect on MAPK phosphorylation and ROS generation, as observed in tissues of *Solanum peruvianum*, *Solanum lycopersicum*, *Nicotiana tabacum*, and *Arabidopsis thaliana*. We hypothesize that methanol acts as a DAMP or DAMP-like molecule in plant defense.

CHAPTER 2: MATERIALS AND METHODS

Cell Bioassay System

S. peruvianum suspension cultured cells were cultivated in Murashige and Skoog (MS) media, with MS vitamins, in 3% (w/v) sucrose with 5 mg/L NAA. BY2 *N. tabacum* suspension cultured cells were cultivated in Murashige and Skoog (MS) media, with MS vitamins, in 3% (w/v) sucrose with 2 mg/L 2,4-D. *S. peruvianum* cultures were used at 7-10 days of age, while BY2 cultures were used at 6-7 days of age. Assays were performed shaken at 150 RPM, in 12-well plates containing 1.5 mL of culture per well. Each sample for collection was represented by two wells (3 mL of culture).

After plating, cells were allowed to equilibrate for an hour. Cultures were then tested for adequate pH before treatment (pH 4.7-5.0), as an indicator of health. Solvents were administered as a pretreatment 1-2 min before the elicitor, and timepoints were collected according to time after elicitor treatment. Elicitors used were flg22 (GenScript, <http://www.genscript.com>), systemin, chitosan and polygalacturonic acid (Sigma-Aldrich, <http://www.sigmaaldrich.com>). Upon collection, culture was aspirated with a 5 mL pipette and separated from media using a vacuum pump, Miracloth and a Büchner funnel. Cells were scraped, then flash-frozen in aluminum foil in liquid N₂.

Arabidopsis Seedling Bioassay System

Seeds were sterilized in ethanol (1 min) followed by vortexing with a 30% commercial bleach solution containing 20% Triton X 100 (5 min), and washed with sterile water ten times. Seeds were vernalized for 48 hrs. and germinated on ½ MS plates with sucrose on a long day cycle. For the assay, 8-10 2- and 4-leaf stage seedlings were selected for timepoints of each treatment. Seedlings were placed into a sterile 24-well plate so that each timepoint sample occupied two wells (4-5 plants/well). Seedlings were incubated on a shaker, plates covered, submersed in 2 mL Gamborg's (2% sucrose with vitamins) overnight.

Each well was treated with 0.5 mL of treatment solution in Gamborg's media. Treatment types and final concentrations included Gamborg's only/"H₂O", 3% methanol, 3 nM flg22, and 3 nM flg22 + 3% methanol, and were given 3 minutes apart. Plants were removed from wells with forceps, gently blotted to remove media, and placed in aluminum foil to be flash frozen in N₂.

Immunoblot Analysis

Frozen plant material was homogenized in extraction buffer containing 50 mM Hepes/KOH (pH 7.6), 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 20 mM b-glycerophosphate, 20% v/v glycerol, 10 µM leupeptin, 1 mM Na₃VO₄, 1 mM NaF, and 1 mM phenylmethanesulfonyl fluoride. Homogenates were centrifuged twice for 10 min at 18,000g at 4°C. Protein concentrations were determined using Bio-Rad protein assay solution (<http://www.bio-rad.com>) using BSA as the standard. Protein aliquots (30 µg) were separated using 10% polyacrylamide gels, and transferred to Immobilon-P PVDF

membranes (Millipore, <http://www.millipore.com>) using a mini trans-blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's recommendations.

After transfer, membranes were blocked in 5% w/v BSA (fraction VII, Fisher Scientific, <http://www.fishersci.com>) in TBS Tween 20 (TBST, 10 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween-20) for 1 hr. at room temperature. Primary antibody used was anti-p44/42 MAP Kinase, 1:2,500 in 5% w/v BSA (ThermoFisher/Pierce, <http://www.piercenet.com>); primary antibody was added to blocking solution and incubated overnight at 4°C. After five washes with TBST, blots were incubated with alkaline phosphatase-conjugated secondary antibody for 1 h at room temperature. The secondary antibody used was monoclonal mouse anti-rabbit alkaline phosphatase-conjugated antibody (1:20,000; Sigma-Aldrich, <http://www.sigmaaldrich.com>). Blots were washed five times with TBST, and then incubated for 5 min with a LumiPhos chemiluminescence detection system (ThermoFisher/Pierce) and visualized using HyBlot CL autoradiography film (Denville Scientific Inc. <http://www.denvillescientific.com>). Protein for loading controls was stained using a MemCod reversible protein stain kit for PVDF membranes (Thermo Fisher/Pierce).

Leaf Disk Luminol Assay

96 leaf disks from *S. lycopersicum* or *S. cheesmaniae* were cut using a cork borer (4 mm), and placed in a Lumitrac 200 96-well plate (Greiner Bio-One, <http://www.greinerbioone.com>) in water overnight. Immediately before reading, wells

were filled with a reagent solution containing 34 $\mu\text{g/mL}$ luminol (Sigma-Aldrich), 20 $\mu\text{g/mL}$ horseradish peroxidase (MP Biomedicals, <http://www.mpbio.com>), and 100 nM flg22 (GenScript) in water. Methanol concentration used was 3%. Readings were taken using a Synergy HT Multi-Mode Microplate Reader (BioTek, <http://www.biotek.com>) in Kinetic Read mode, set to read endpoint luminescence in relative luminescence units (RLU). Assay lasted 45 min and readings were taken every 64 sec for a total of 45 reads. Data was collected using Gen5 Data Analysis Software (BioTek).

Feeding Assay System For RIDA Analysis

Two-leaf stage *S. lycopersicum* plants were excised at stem base, and placed in groups of three in 1.5 mL of treatment solution for 1 hr. Treatment doses were solved in sterile nanopure water and included 7 nM flagellin or 10 nM systemin, 3% solvent, solvent and elicitor combined, or water alone. The plants were then placed in water alone for a 24 hr. incubation period. Leaves were pressed for leaf juice; 5 μL was collected from each 3-plant sample for addition to RIDA plate wells. RIDA plates contained agarose gel with anti-PI II goat antiserum from Spring Valley Laboratories (Woodbine, MD). Rings formed by antiserum precipitate were developed for analysis using a 0.75% acetic acid wash. PI II accumulation was calculated using the method described in Ryan's 1967 paper (27). Results were evaluated for statistical significance using a two-tailed Student T-Test with unequal variances.

CHAPTER 3: RESULTS

We originally encountered the effect of ethanol on MAPK6 phosphorylation in *S. peruvianum* cells while doing experiments with combined systemin and epibrassinolide treatments. In our initial experiments, epibrassinolide was given in 50 μ L of ethanol per 1.5 mL of cell culture, which resulted in a 3.2% concentration of ethanol in each well. The treatment prolonged MAPK6 phosphorylation past its 10-30 minute prime to at least 45 minutes. We did not expect the ethanol to have any effect on the result, therefore controls for ethanol combined with systemin alone were not initially included in the experiments. This led us to believe the epibrassinolide was responsible for the prolonged phosphorylation of MAPK6.

We eventually found this was not the case, and that ethanol alone intensified the phosphorylation caused by systemin at later timepoints. After several replicates confirming the effect with and without epibrassinolide, it was decided that the concentration of ethanol necessary for this effect should be investigated, and whether the solvent affected other elicitors' effect. We found that a concentration of 3% ethanol consistently extended the phosphorylation of MAPK6 caused by flg22 or systemin. Concentrations as low as 2-2.5% could also have the same effect (Fig. 4.1a). At concentrations lower than these however, the effect proved to be highly variable between replicates.

We hypothesized that perhaps ethanol could be a parallel alert mechanism to elicitor perception during bacterial growth in the presence of bacterial anaerobic metabolism. We then also considered that methanol, being similar in structure to ethanol and a byproduct of bacterial invasion of the plant cell wall, could have a similar effect on MAPK6 phosphorylation in the presence of flg22. We found that methanol prolonged phosphorylation in the presence of flg22, systemin and chitosan (Fig. 4.1b). Since chitosan showed an atypical response to the methanol treatment, we tested duplicate samples with the higher concentrations (2.5% and 3%) and found that the 2.5% methanol treatment yielded a variable result. Methanol and ethanol were therefore found to have a concentration dependent effect on MAPK6 phosphorylation in *S. peruvianum* upon application of an elicitor. We improved the resolution of the timecourses in *S. peruvianum* to show the gradual activity of both solvents in the presence of flg22, systemin, and chitosan (Fig 4.2a, b, c). Methanol and ethanol alone rarely caused MAPK6 phosphorylation throughout the timecourse, except a few minutes after treatment.

After showing that the effect of the two solvents was similar in the presence of multiple elicitors, we hypothesized that this effect might occur in other members of the Solanaceae family. We therefore tested the effect in a BY2 (*N. tabacum*) cell line. We found results similar to those seen in the *S. peruvianum* cell line for flg22 (Fig. 4.3a) and chitosan (Fig. 4.3b). Since tobacco cells are not sensitive to systemin, we replaced systemin with polygalacturonic acid, or PGA (Fig. 4.3c), testing only with methanol. We obtained a similar result to that seen with flg22 and chitosan treatments. We therefore

concluded that the effect of methanol and ethanol on the phosphorylation of MAPK6 observed in *N. tabacum* is similar to that seen in *S. peruvianum*, showing extended phosphorylation after elicitor treatment.

These experiments were carried out using heterotrophic cells. It was therefore important to test whether methanol also alters MAPK phosphorylation in photosynthetic plant tissues. We treated 2-4 leaf stage *A. thaliana* seedlings with a flg22 and methanol double treatment. After five replicates we found that the MAPK phosphorylation induced by the double treatment at 45 min was higher than that of samples treated with flg22 alone (Fig. 4.4). In our replicates we did not always see phosphorylation differences as striking as in the cell experiments, but we consistently saw more intense signals for double treated plants at later timepoints, sometimes at both 30 and 45 min. We therefore concluded that methanol can be perceived by plants through an unknown mechanism that results in a modulation of MAPK phosphorylation in the presence of an elicitor.

Carlton Bequette helped us perform ROS assays using Luminol and *S. lycopersicum* (Figure 4.5) and *S. cheesmaniae* (Figure 4.6) leaf disks. Leaf disks were treated with flg22 and methanol, to test whether the ROS burst induced by flg22 was affected by the presence of methanol. Flg22-induced ROS production follows a curve similar to that followed by MAPK6 activity, with strong intensity 5-15 min after treatment that tapers off by 45 min. In double treated samples, the peak of this curve is shifted by several minutes, and by ~35 min, the relative LUM of the double treated leaf disks was two fold higher than flg22 alone.

Throughout the course of the study we were interested in finding out whether the elicitor and solvent-induced MAPK phosphorylation would have any effect on plants' downstream defense responses. We decided to measure this through analysis of proteinase inhibitor II production, which is an important output response to the perception of herbivory in tomato. We used the RIDA (radial immunodiffusion assay) method with 2-leaf stage tomato plants. The plants were treated with methanol or ethanol doses with systemin or flg22. RIDA results were inconclusive. While some experiments clearly suggested that the double treatment caused an enhancing effect in PI II production, others suggested that the double treatment was suppressing PI II production or having no effect (Table 4.1). We concluded that either the treatment method interfered with the signalling involved in PI II production, or that other unknown factors might be modulating the plants' response to the solvent.

CHAPTER 4: DISCUSSION

Our results suggest that methanol and ethanol modulate the activation of MAPK6 in the presence of an elicitor. This is most obvious at later timepoints in our experiments, when the phosphorylation in samples treated with elicitor only was much lower than those treated with both a solvent and an elicitor. However, at earlier timepoints, methanol suppresses this phosphorylation. Therefore, methanol appears to cause a shift in the phosphorylation timing in the presence of an elicitor. At the moment, implications for a short forward shift in MAPK6 activation timing are unknown.

ROS burst is an initial component of signaling in plant innate immunity. The generation of ROS upon elicitor perception is involved in transcriptional changes that regulate the early response to pathogens. Temporal and amplitude shifts in the ROS burst may have implications for the timing and intensity of the early response in plants. In our experiments, we observed a shift in timing of the ROS burst in *S. lycopersicum* and a shift in both amplitude and timing of the ROS burst in *S. cheesmaniae*. Although the effects of these shifts on plants' output responses to flg22 are unknown, these results suggest that methanol may modulate other elicitor-induced signaling modules in the plant cell.

We were not able to demonstrate that methanol has effects downstream from MAPK signaling in our experiments. The results we obtained on PI II production are flawed however, because the feeding assay system requires the stem to be cut in order to deliver treatment. This method is problematic because within a 12-24 hr period, wounding can cause an increase in PME activity that generates methanol. Although we did not measure the release of methanol from the plants used in these experiments, it is highly probable that methanol release from wounding combined with exogenous methanol application may have significantly impacted the consistency of the results. These results should therefore not preclude methanol from being studied as a DAMP. Since PME has been shown to be responsive to biotic stress, and considering the potential for cell wall integrity-related signaling in plants, a byproduct of PME activity should be studied as a possible signaling component.

A better understanding of the gene expression effects of methanol-induced prolonged MAPK6 activation would clarify methanol's DAMP-like role. An *A. thaliana* study by Anderson et al. in 2011 has shown that MAPK phosphatase mutant *mkp1* has increased resistance to *Pseudomonas syringae* pv. tomato (*Pto*) DC3000, in addition to having enhanced ROS production and increased levels of early response gene transcripts. After exposure to MAMPs, ERF, WRKY53 and WRKY40 transcripts were more abundant than in the WT control. The study shows that the MKP1 mutation's effects on immunity are MAPK6 dependent, suggesting that defense-related gene activation can be enhanced by the prolonged activity of MAPK6 (1). In the future, examining the transcript levels of early response genes upregulated by MAPK6

activation would reveal whether the effect of methanol actually alters the defense response, and not just the phosphorylation of MAPK6.

The importance of the timing of MAPK activation in regulating downstream effects of a transduced signal is also evidenced by other examples in eukaryotic cells. In 2001, Kao et al. found that treatment of PC12 nerve cells with different growth factors had different effects on the duration of the phosphorylation of ERK, a MAPK. Treatment with epidermal growth factor (EGF) transiently activated ERK, and caused PC12 cells to proliferate. On the other hand, treatment with nerve growth factor (NGF) activated ERK in a sustained manner, causing PC12 cells to differentiate (14). Based on our understanding of the importance of timing in MAPK phosphorylation, we believe the effect of methanol on signal transduction may have an impact on downstream responses to elicitors.

In our experiments, the altered phosphorylation of MAPK6 suggests that plant cells can detect methanol in the presence of a defense elicitor. Since methanol is an abundant byproduct of cell wall degradation, and it modulates MAPK6 activity, it behaves similarly to a DAMP. This finding suggests that for plants, methanol might have a role in amplifying or changing the downstream response to the elicitor during increases in plant or pathogen PME activity.

DAMP-like activity of methanol could be advantageous in a cellular microenvironment, such as two adjacent cell walls, in which a methanol-sensing mechanism is in place. If methanol levels could be perceived as abnormally high for the growth status of a small group of cells, this could alert those cells to the presence of

pathogen CWDEs. This ability would be especially relevant if the presence of small amounts of MAMPs, combined with a critical concentration of methanol, could tip the cell off that a pathogen is invading.

Small amounts of MAMPs from pathogens, regardless of their virulence, may be ubiquitous for plant tissues and may not always warrant a response. Methanol might act as a simple indicator that MAMPs present are response-worthy, making responses less wasteful. If methanol levels are detected as abnormal amongst a group of cells, the solvent might amplify MAPK6 activation in response to MAMPs. Our results suggest that methanol should be studied in the context of a DAMP in the future, as it might be aiding cell wall integrity signaling to the energetic advantage of the plant.

A

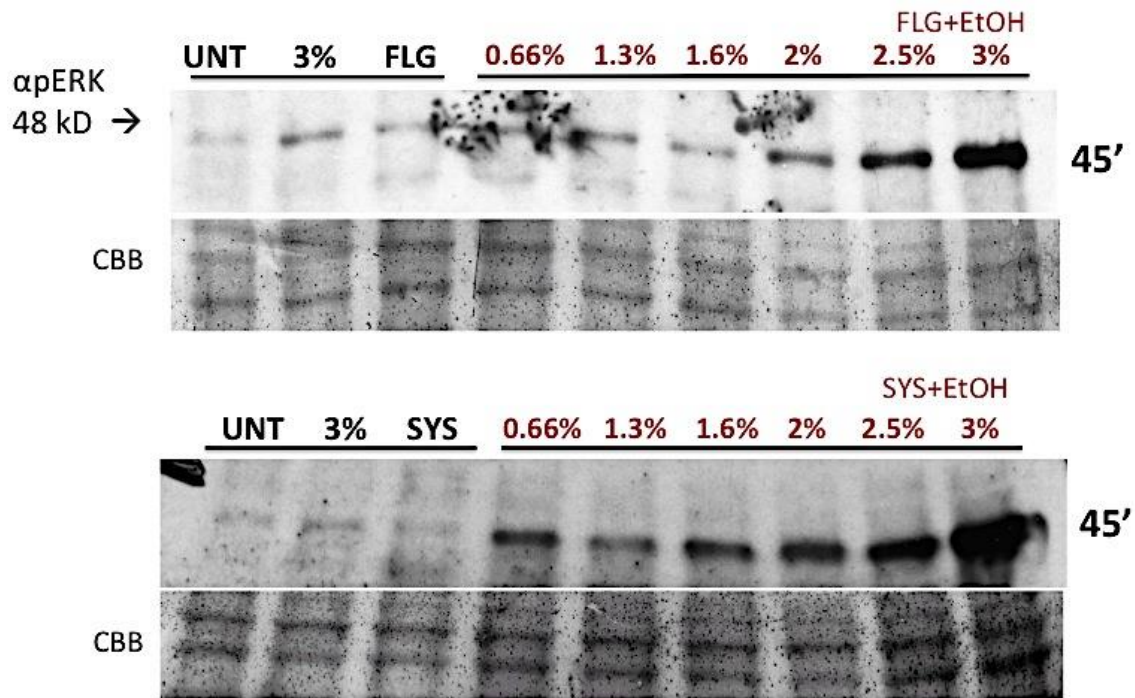
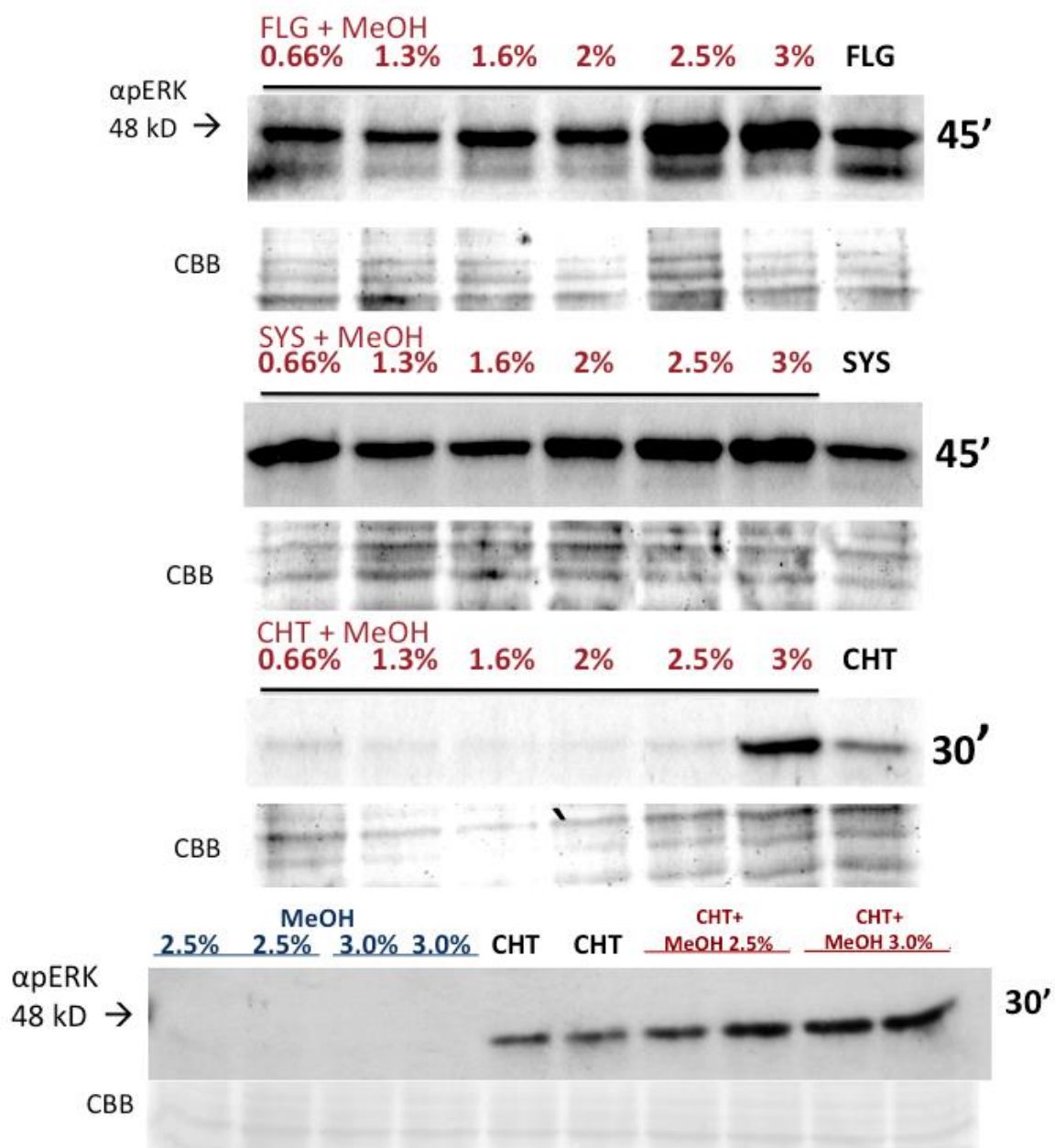


Figure 4.1. Ethanol and methanol have a concentration dependent effect on MAPK6 phosphorylation in the presence of an elicitor. *S. peruvianum* cells were treated with 3% solvent, then with elicitor after 1-2 min. Double-treated cells show phosphorylation longer than those treated with elicitor alone. Samples were collected at 45 min. after treatment, except for those treated with chitosan, which were collected at 30 minutes after treatment. **A**, Ethanol prolongs the phosphorylation by 0.7 nM flg22 ("FLG") and 1 nM systemin ("SYS"). Controls include ethanol alone (3%), elicitor alone, and untreated (UNT). **B**, Methanol prolongs the phosphorylation of MAPK6 by 0.7 nM flg22 ("FLG"), 1 nM systemin ("SYS") or 1.7 µg/mL chitosan ("CHT"). Second panel at bottom shows variation in double treatments with 2.5% methanol and chitosan, as compared to results in first panel.

B



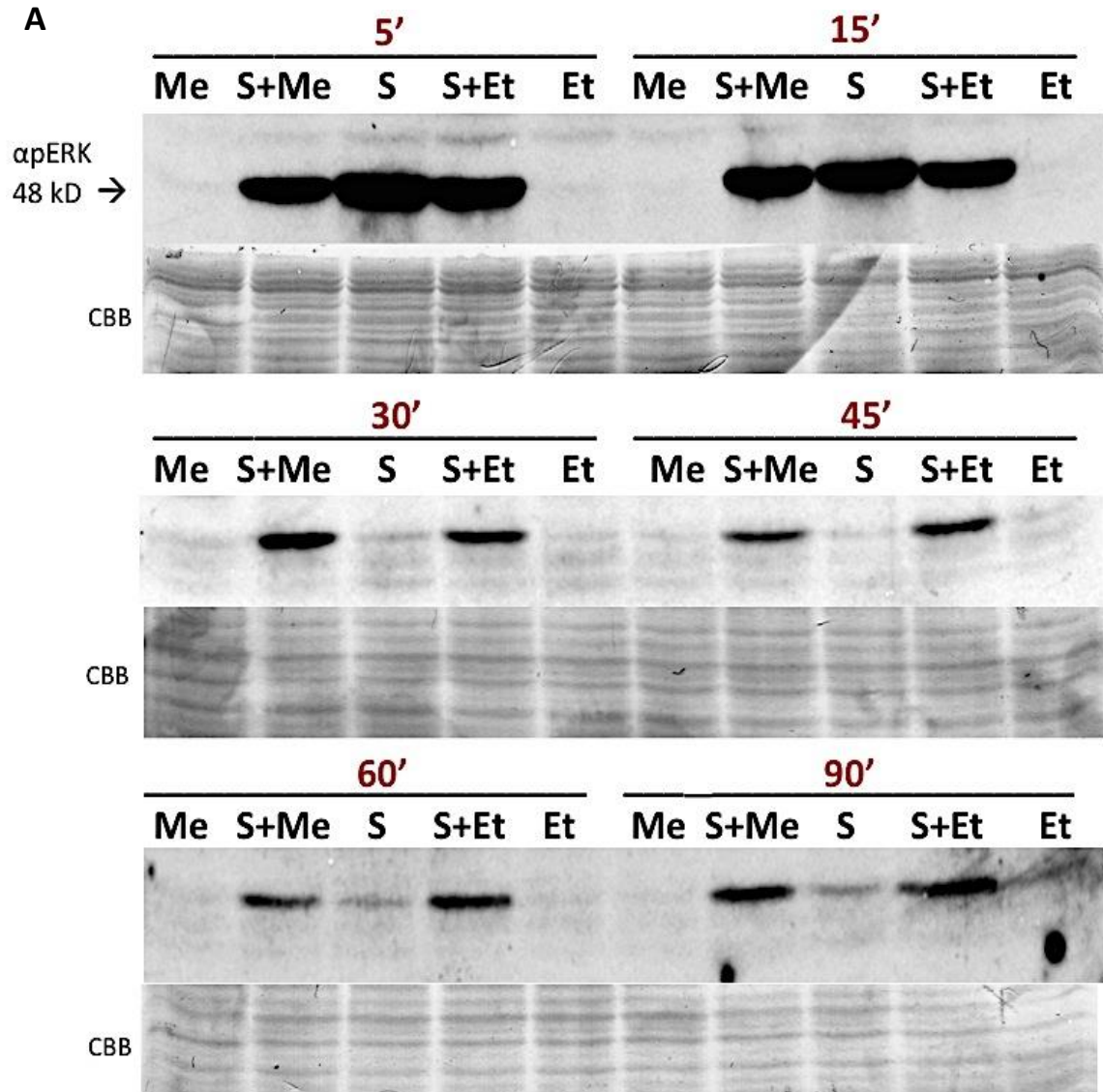
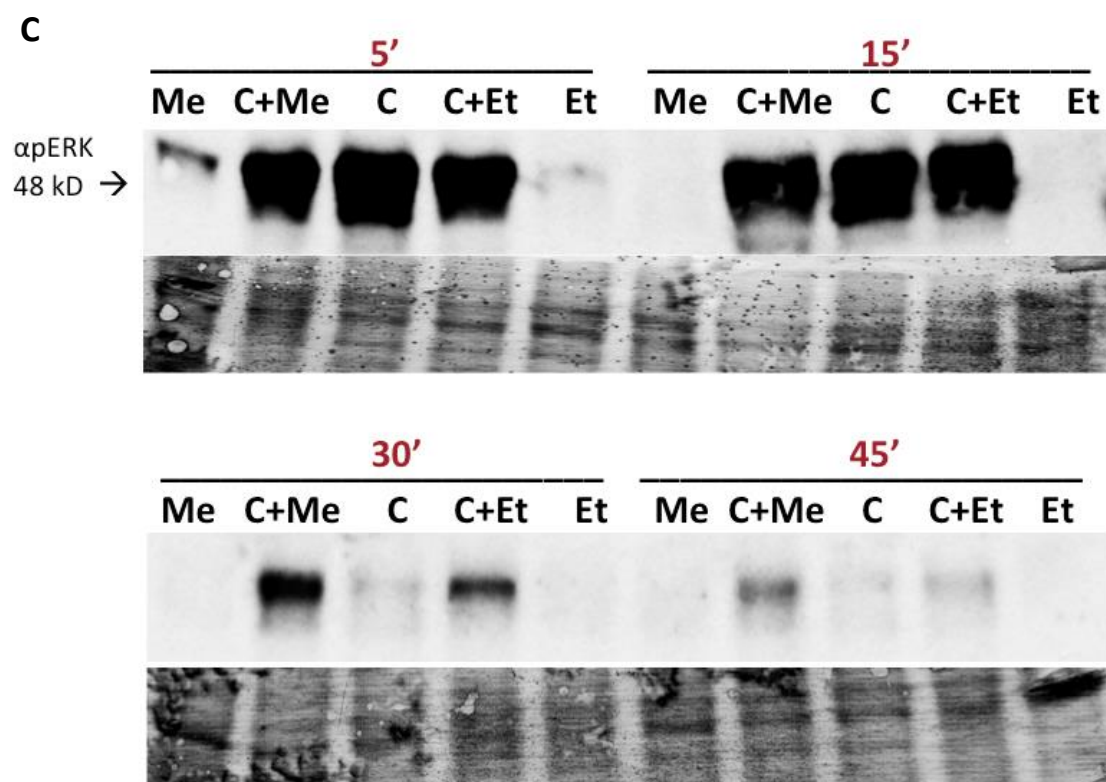
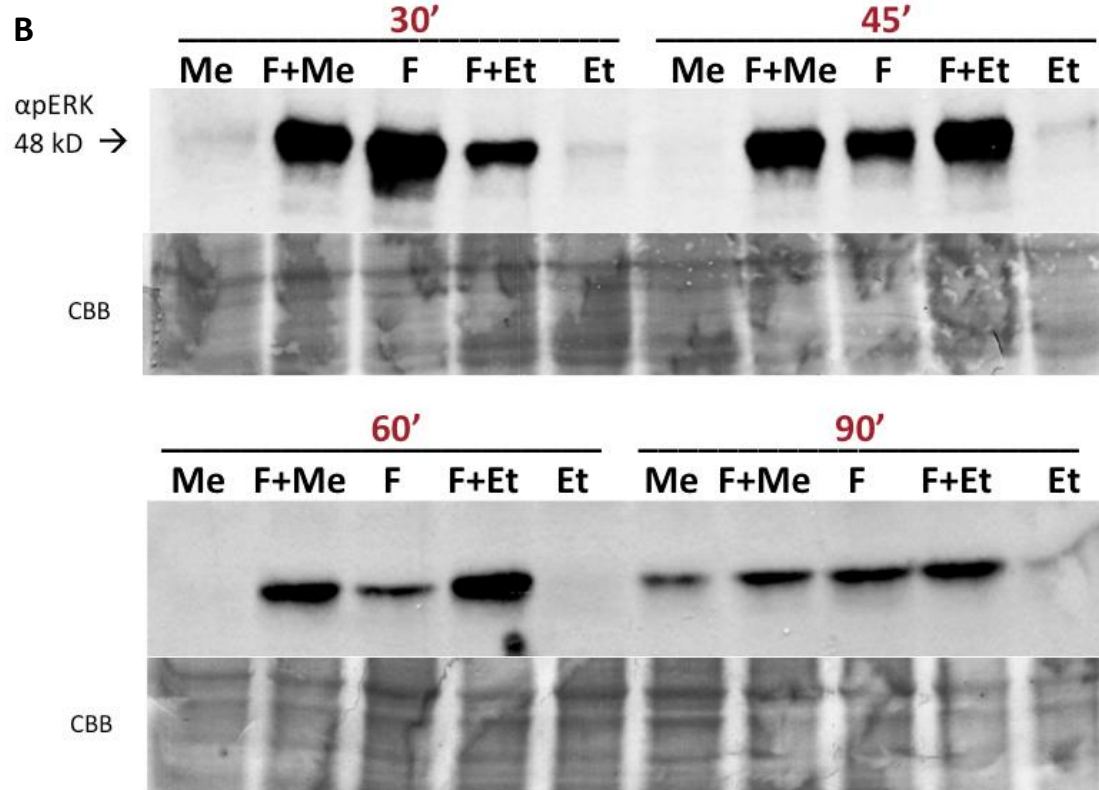


Figure 4.2. Tomato (*Solanum peruvianum*) cell culture timecourse analysis of MAPK6 phosphorylation following treatment with elicitors and 3% methanol or ethanol. Double-treated cells show phosphorylation longer than those treated with elicitor alone and suppression at earlier timepoints. Cells were treated with solvent first, then with elicitor 1-2 min. after. Controls include methanol ("Me"), ethanol ("Et"), and elicitor alone. **A**, Timecourse with 1 nM systemin ("S"). **B**, Timecourse with 0.7 nM flg22 ("F"). **C**, Timecourse with 1.7 µg/mL chitosan ("C").



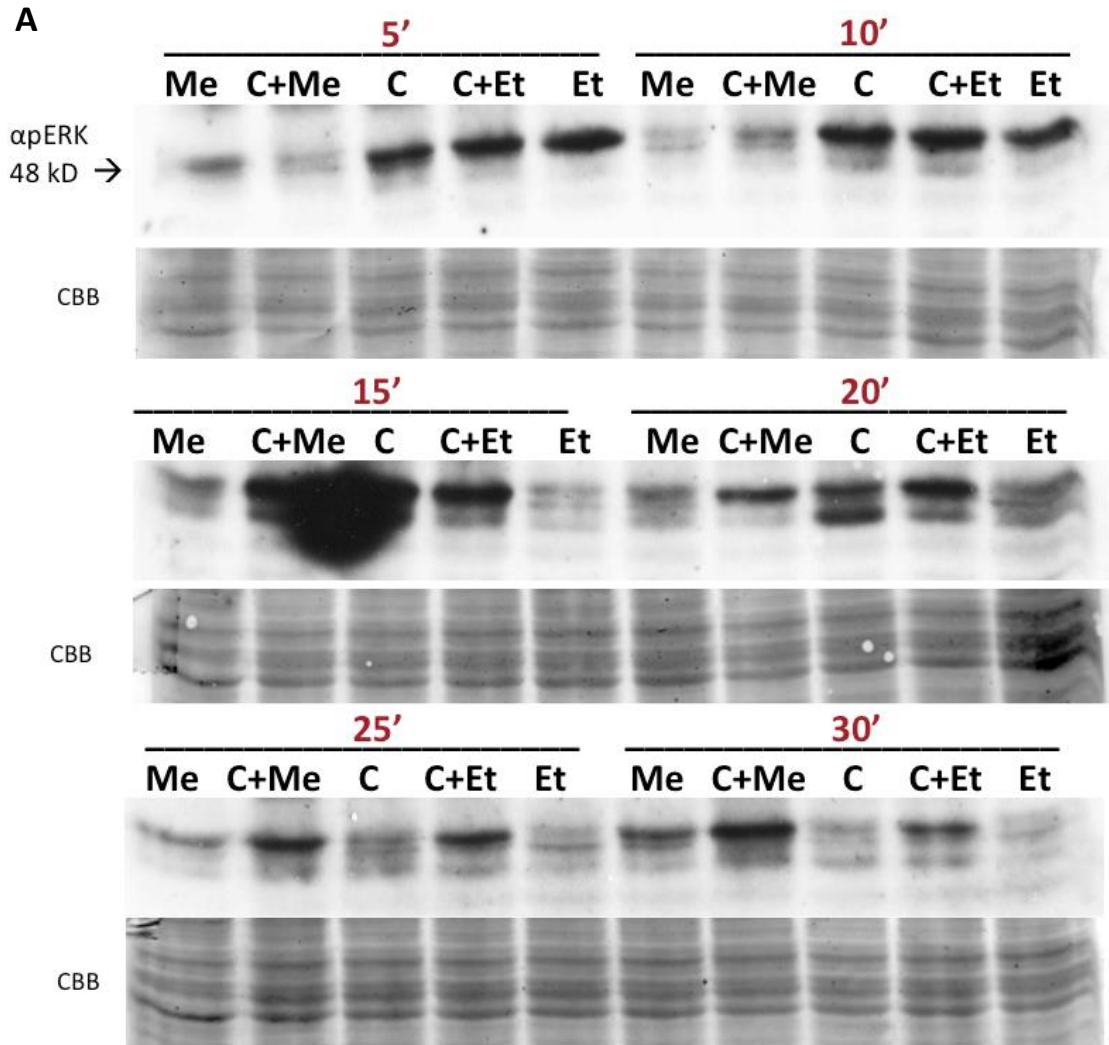
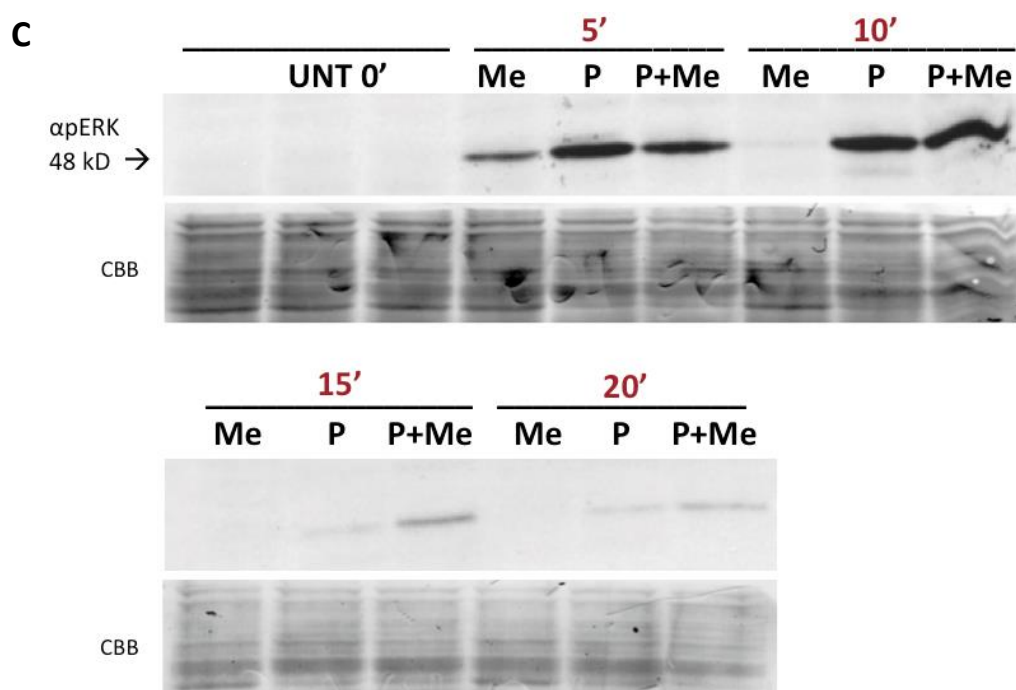
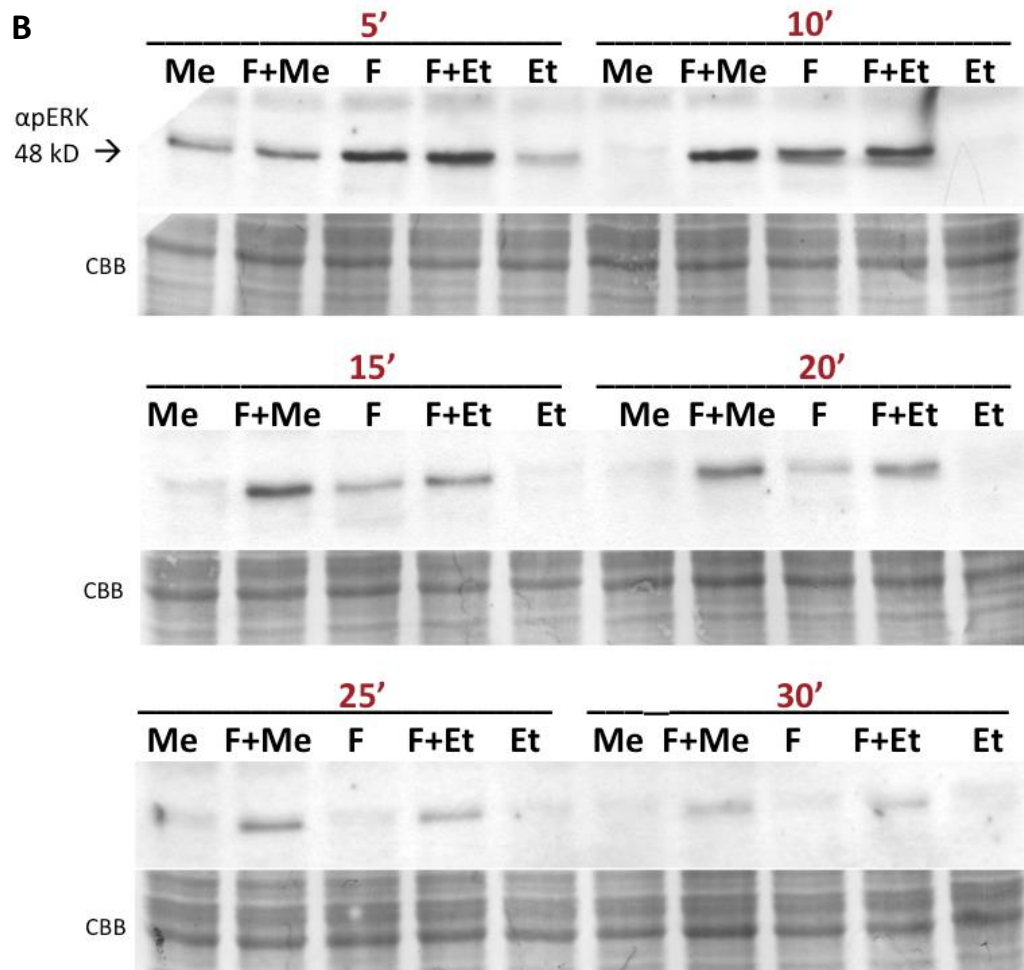


Figure 4.3. Tobacco (*Nicotiana tabacum*) cell culture timecourse analysis of MAPK6 phosphorylation following treatment with elicitors and 3% methanol or ethanol. Double-treated cells show phosphorylation longer than those treated with elicitor alone and suppression of phosphorylation at earlier timepoints. Cells were treated with solvent, then with elicitor 1-2 min. after. Controls include methanol (“Me”), ethanol (“Et”), and elicitor alone. **A**, Timecourse with 1.7 $\mu\text{g}/\text{mL}$ chitosan (“C”). **B**, Timecourse with 30 nM flg22 (“F”). **C**, Timecourse with 20 $\mu\text{g}/\text{mL}$ PGA (“P”) and methanol only (“Me”).



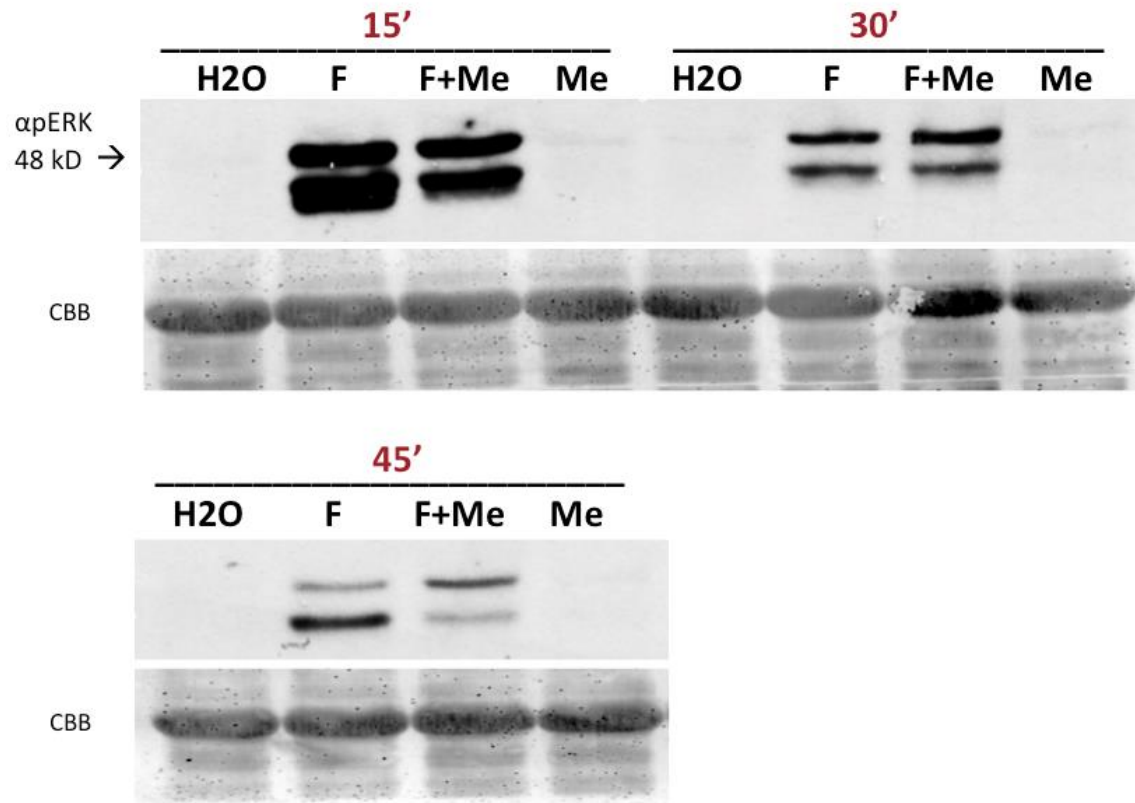


Figure 4.4. *A. thaliana* seedling timecourse analysis of MAPK6 phosphorylation following treatment with 3.5 nM flg22 and 3% methanol. Double-treated plants show phosphorylation more strongly than those treated with elicitor alone at later timepoints. Cells were treated with solvent and elicitor simultaneously. Controls include “H₂O”, which used Gamborg’s medium alone, methanol (“Me”), and flg22 (“F”) alone. All treatments were solved in medium when added to wells.

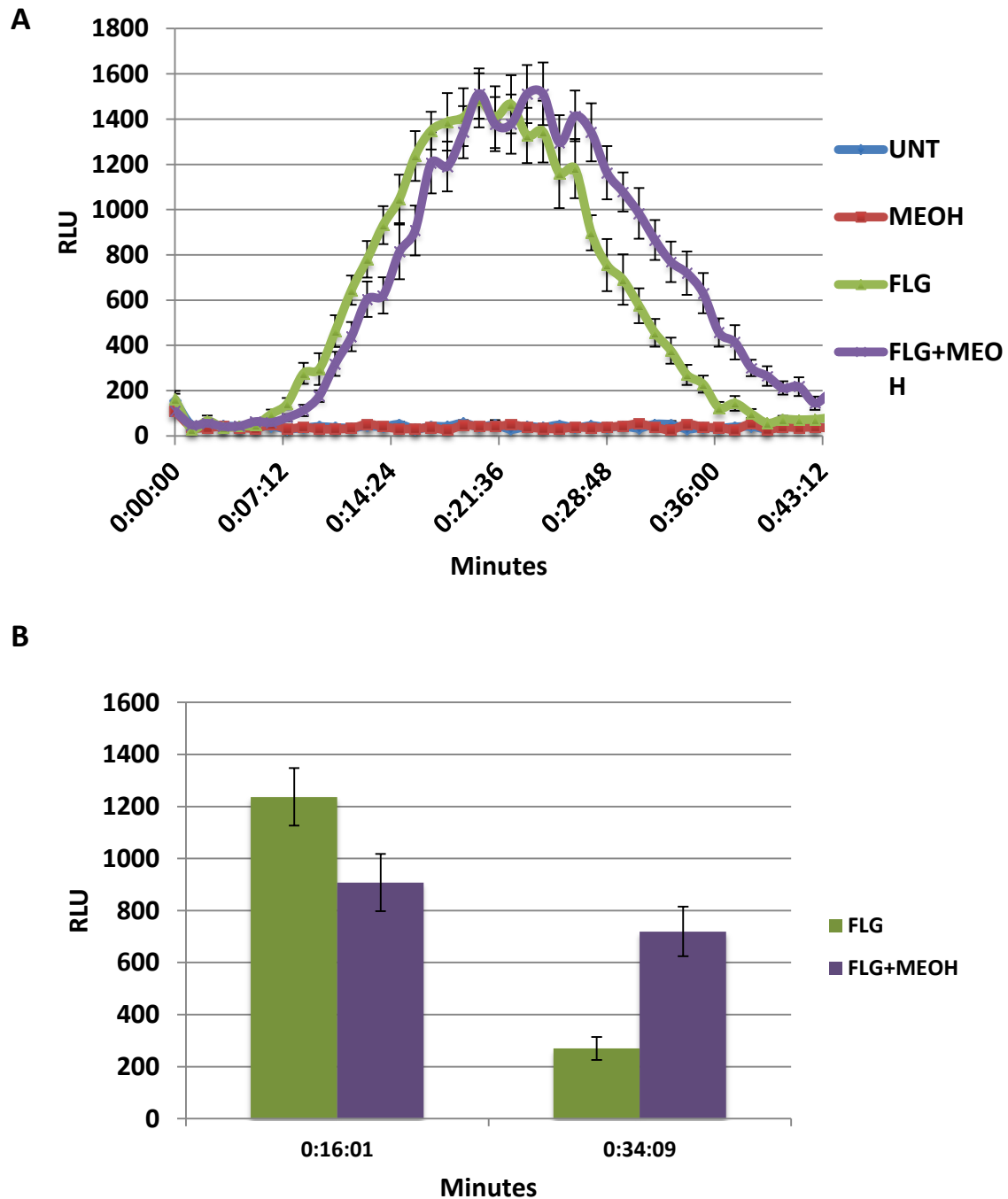


Figure 4.5. ROS burst in *S. lycopersicum* (tomato, Rio Grande) leaf disks after flg22 and methanol treatment. **A**, ROS burst is followed over 45 min using relative luminance units (RLU) readings in a luminol assay. **B**, Difference in ROS burst timing for double treated leaf disks is most pronounced at early and late timepoints.

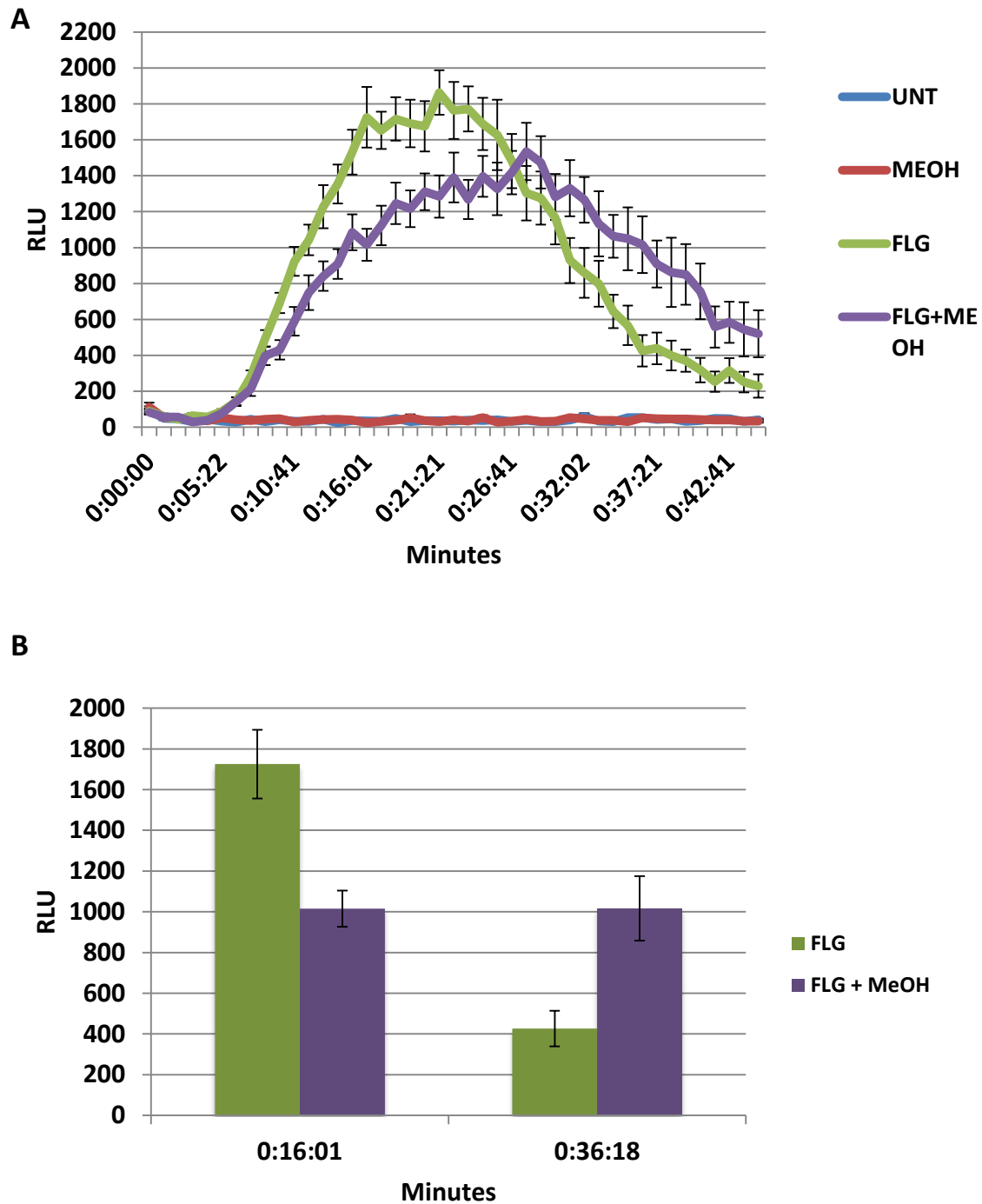


Figure 4.6. ROS burst in *S. cheesmaniae* (wild tomato) leaf disks after flg22 and methanol treatment. **A**, ROS burst is followed over 45 min using relative luminance units (RLU) readings in a luminol assay. **B**, Difference in ROS burst timing for double treated leaf disks is most pronounced at early and late timepoints.

Table 4.1 RIDA analysis of PI II production after double treatment with ethanol or methanol, and flagellin or systemin, shows inconsistent effect

	Methanol	Ethanol
Systemin	5 synergistic	1 synergistic
	9 suppressive	0 suppressive
	11 neutral	5 neutral
Flagellin	6 synergistic	6 synergistic
	4 suppressive	0 suppressive
	9 neutral	4 neutral

In double treated plants, “synergistic” results showed PI II production higher than that of plants treated with solvent alone and plants treated with elicitor alone combined. “Suppressive” results showed PI II production lower than that of plants treated with solvent alone and plants treated with elicitor alone combined. “Neutral” results showed neither effect.

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