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RESEARCH PAPER

A citrus abscission agent induces anoxia- and senescence-related gene expression in *Arabidopsis*

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Abstract

The mechanisms of negative effects of 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP), a pyrazole-derived plant growth regulator used as a citrus abscission agent, were explored in *Arabidopsis* by integrating transcriptomic, physiological, and ultrastructural analyses. CMNP promoted starch degradation and senescence-related symptoms, such as chloroplast membrane disruption, electrolyte leakage, and decreased chlorophyll and protein content. Symptoms of plant decline were evident 12 h after CMNP treatment. Microarray analysis revealed that CMNP influenced genes associated with stress, including those related to anoxia, senescence, and detoxification. Sucrose treatment arrested CMNP-induced plant decline. The results demonstrate that the plant response to CMNP shares common elements with various stresses and senescence at physiological and molecular levels.

Key words: Electrolyte leakage, microarray analysis, plant decline, stress.

Introduction

The compound 5-chloro-3-methyl-4-nitro-1*H*-pyrazole (CMNP) is a plant growth regulator that advances abscission in mature *Citrus sinensis* fruit when applied to tree canopies and also plant decline in *Arabidopsis thaliana* when applied as a root drench (Alferez *et al*., 2005). CMNP acted as an uncoupler of energetic membranes, depleted total ATP content, and increased phospholipase A_2 (PLA_2) and lipoxygenase activities and production of downstream lipid signalling compounds. Moreover, CMNP reduced fruit detachment force, promoted starch breakdown and peel colour change by inducing total carotenoid accumulation and chlorophyll degradation, increased electrolyte leakage and membrane degradation, and induced protein catabolism in citrus fruit. In summary, CMNP promoted wounding-like symptoms in peel, accelerated senescence, and advanced abscission in citrus fruit (Alferez *et al*., 2006). Dinitrophenol also reduced mature fruit detachment force and accelerated abscission when applied to citrus canopies (Alferez *et al*., 2005). This implies that low molecular weight compounds with uncoupling activity may promote abscission.

Transcriptome-level analyses indicate that the plant response to various stresses involves changes in numerous metabolic and signal transduction pathways (Cheong *et al*., 2002; Kreps *et al*., 2002; Loreti *et al*., 2005). Application of plant growth regulators and other xenobiotic materials to plants alters gene expression associated with abiotic and biotic stresses and detoxification (Zhu and Wang, 2000; Ekman *et al*., 2003; Mezzari *et al*., 2005). Besides compound-specific responses, stress changes carbohydrate content and alters expression of genes involved in sugar metabolism (Provart *et al*., 2003). Under aerobic conditions, catabolism of carbohydrates involves metabolism of pyruvate by pyruvate dehydrogenase (PDH) and activation of cellular respiration. If oxidative phosphorylation is impaired by uncoupling or anoxic conditions, metabolism is switched to fermentation, and pyruvate is converted to acetaldehyde by pyruvate decarboxylase (PDC) and then to ethanol via

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Abbreviations: ADH, alcohol dehydrogenase; CHI, chitinase; CMNP, 5-chloro-3-methyl-4-nitro-1*H*-pyrazole; FK, fructokinase; GLU, β-1,3 glucanase; GDH, glutamate dehydrogenase; PLA_2, phospholipase A_2; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; RCO, Rubisco, small subunit; TPS, trehalose phosphate synthase.

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alcohol dehydrogenase (ADH) (Smeekens, 2000). Under different biotic and abiotic stresses, including anoxia, hexokinases in general, and particularly fructokinases (FKs) and glucokinases, are activated, regulating sugar entry into glycolysis (Fox *et al*., 1998). In addition to anoxia-induced changes in carbohydrate metabolism, loss of complex carbohydrate and transient accumulation of soluble sugar is a feature of senescence (Buchanan-Wollaston *et al*., 2003).

Wounding alters local and systemic gene expression associated with carbohydrate metabolism at the wound site and throughout the plant (Delessert *et al*., 2004; Quilliam *et al*., 2006). Abscission occurs in young developing fruitlets if they unsuccessfully compete for carbohydrate (Gomez-Cadenas *et al*., 2000). Exogenous application of sugar stimulated the early phase and inhibited the late phase of senescence (Paul and Pellny, 2003), reduced fruitlet abscission (Iglesias *et al*., 2003), and enhanced tolerance to anoxia (Loreti *et al*., 2005). The role of sugar in reaction and recovery of plants to various stress conditions may be 2-fold: to provide carbon for reconstructive metabolism and to act as a signal eliciting downstream responses (Rolland *et al*., 2002).

In an attempt to understand further how responses to CMNP operate, some physiological- and transcriptional-level responses of *Arabidopsis* leaves to root application of CMNP were studied. Pyrazole, the parent compound of CMNP, is a constituent of some herbicides that are rapidly absorbed by roots of target plants and impact energetic machineries in plant cells (Duke *et al*., 2000). In addition to the uncoupling effect of CMNP, many substituted pyrazoles are also ADH inhibitors (Dahlbom *et al*., 1974). Inhibition of ADH by CMNP would reduce NAD-linked production of ATP in glycolysis and further reduce cellular energy supply, compromising cellular membrane integrity (Rawyler *et al*., 2002). We hypothesized that CMNP uncoupling action would modify expression of genes associated with carbon metabolism and energy supply, thus triggering plant response in *Arabidopsis*. The results demonstrate that genes associated with stress are influenced by root application of CMNP, and that providing a carbon source altered symptom development.

Materials and methods

Plant material and treatments

Rossette leaves at positions 5 and 6 (stages 3.70–3.90, Boyes *et al*., 2001) from 4-week-old *Arabidopsis thaliana* adult plants were used. Seeds were stratified by imbibing at 4 °C for 48 h and then transferred to 4 cm deep trays containing potting medium (Metro Mix 500). Plants were grown in growth chambers for 2 weeks at 20 °C (10 h light at 300 μmol m^{-2} s^{-1} and 14 h dark) and then transferred to 26 °C under incandescent lamps (300 μmol m^{-2} s^{-1}) with a 14 h light/10 h dark cycle for 1 week before treatment. Individual plants were removed from the tray and roots were immersed in beakers containing 50 ml of test solutions under continuous light (300 μmol m^{-2} s^{-1}) at 26 °C. Leaves for soluble sugars, starch, total chlorophyll, and total protein analysis were harvested periodically up to 16 h after treatment. For electron microscopy, microarray, and real-time PCR analysis, samples taken at 0, 1, 3, and 6 h after treatment were used. Electrolyte leakage measurements were taken in leaves for periods up to 24 h after treatment. All test solutions contained Tween-20 (0.01%) as adjuvant.

Electron microscopy, electrolyte leakage, starch, and soluble sugar determination

For electron microscopy, mature leaves were excised from plants treated with 1.5 mM CMNP or water alone (control) and prepared as previously described (Burns *et al*., 1992). Soluble sugars and starch were quantified as described by Somogy (1952) with some modification. *Arabidopsis* leaves were dried at 60 °C for 3 d and ground to a powder. Samples (100 mg DW) were extracted with 80% ethanol and centrifuged twice for 1 min at 1000 rpm. To recover soluble sugars, HCl was added (1/100, v/v) to the supernatant and boiled for 10 min. For starch determination, pellets were treated overnight at 30 °C with a solution of 1 M MES (pH 5.0) and amyloglucosidase. Sample absorbance was measured at 520 nm in a spectrophotometer and compared with a standard curve of known glucose concentrations. Sugar and starch contents were calculated and expressed as a percentage of the control. Two biological replicates were used and sugar determinations were made in triplicate. Electrolyte leakage was determined in excised leaves immersed in 1.5 mM CMNP or water solutions, and measured as described (Alferez *et al*., 2006).

Chlorophyll and protein quantification

Leaves were collected for various times up to 12 h after treatment application. Chlorophyll was extracted with acetone and 0.1 g sodium carbonate at 4 °C for 1 d. Extract absorbance was measured at 645 nm and 663 nm, and chlorophyll content calculated using the Moran equation (Moran, 1982). Total protein content in extracts was measured by the dye binding method (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard (Bradford, 1976).

Microarray printing, probe labeling, hybridization, and data analysis

The *Arabidopsis* cDNA array was generated from the Arabidopsis Biological Resources Center's Arabidopsis bacterial clone set (www.tigr.org). Microarray slides were printed at the Keck Foundation Biotechnology Resource Laboratory, Yale University as described by Ma *et al*. (2001). Each slide contained two identical arrays, each containing 11 300 expressed sequence tags (ESTs; http://www.crec.ifas.ufl.edu/burns/PDF/supplement1.pdf) representing approximately 6000 unique genes.

Total RNA from samples treated with 1.5 mM CMNP or water was extracted from the leaves of 3-week-old *Arabidopsis* plants using phenol–chloroform. Total RNA was used as a template to synthesize Cy-3- or Cy-5-conjugated deoxy CTP-labelled cDNA probes with the CyScribe first strand cDNA labelling kit (Amersham Biosciences, USA) following the manufacturer's instructions. Three biological replicates provided three independent RNA extractions used for probe labelling, and three slides, each containing two duplicate arrays, were used for each comparison. One array on each slide was hybridized with one labelled sample pair; the remaining array was hybridized with the same sample pair labelled by swapped dyes. Slide pre-hybridization, probe hybridization, slide washing, and scanning were performed as described

by Zhong and Burns (2003). Spot intensities were quantified with ScanAlyze software (Eisen *et al*., 1998; http://rana.lbl.gov/EisenSoftware.htm). Raw data generated by ScanAlyze were analysed by using a Visual Basic program called ArrayTeller (Zhong and Burns, 2003). The correlation coefficient of signals between different repetitions was calculated, and only replicates whose values were higher than 0.92 were considered for this study.

Sucrose effects on symptoms of CMNP-treated plants

For root dip treatments, the CMNP concentration of 1.5 mM was chosen based on progression of plant decline symptoms within a 12 h time span. Adult plants were treated with root dips of 1.5 mM CMNP, 1.5 mM CMNP+2% sucrose, 2% sucrose, or water alone for 12 h. After 12 h, plants were observed and symptoms documented.

Real-time PCR analysis of gene expression

Total RNA was prepared as described above, and contaminating DNA removed with RNase-free DNase (Qiagen). Quantitative PCR was performed with an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the SYBR Green PCR kit (Applied Biosystems). Dilutions (10–1000-fold) of RNA were used in 25 μl reaction volumes to calculate the real-time efficiency of each sample. Relative expression ratios of target genes (*GDH1*, *RCO*, *CHI*, *GLU*, *ADH1*, *PDC1*, *PDH*, *FK1*, and *TPS*) were calculated in comparison with that of a constitutively expressed reference gene (ribosomal *18s*) by the C_T method using software from the ABI PRISM 7500 Sequence Detection System. To achieve comparable amplification of cDNA templates for *r18s* and target genes, RNA concentrations were diluted by 1/10 000 when amplified using *r18s* primers. Expression of *r18s* was normalized to a value of 1.0, and the relative expression of target genes was compared with the normalized value of *r18s*. The following specific primers were used: *GDH1* (forward 5′-CAGGGCAGCGTTTTGTCAT-3′ and reverse 5′-GCTTTGCCGCCCAAGAA-3′); *RCO* (forward 5′-CGACATTACTTCCATCACAAGCA-3′ and reverse 5′-GGCCACACCTGCATGCA-3′); *CHI* (forward 5′-GCTTGTCCCGCAAATGGT-3′ and reverse 5′-CGCCTCGTCGCTTGGA-3′); *GLU* (forward 5′-TGGGTGAGAGCAACATGCA-3′ and reverse 5′-TGGCGTAGGTCAAGAGCAAGA-3′); *ADH1* (forward 5′-GGAGGAGCAAGAGGGATAGGA-3′ and reverse, 5′-ACTATCACATACGCGCCATTCTC-3′); *PDC1* (forward 5′-AAGATTTCCAAACTCAGGCAAGAG-3′ and reverse, 5′-ATGCAAGCCAAACAAAAACGA-3′); *PDH* (forward 5′- GGCGAGCACATTACCATCCT-3′ and reverse, 5′- TTGCTGCCTGCATCACATG-3′), *FK1* (forward 5′-GAGAGGCGCTTCTATTTGCAA-3′ and reverse 5′-GGCATCGCCGGTATCG-3′); and *TPS* (forward 5′-TGGGATGAATCTTGTCAGTTATGAG-3′ and reverse 5′-TGAGAATGAGGACGCCCTTT-3′).

Results

Physiological consequences of CMNP application

Transmission electron microscopy of CMNP-treated plants revealed that chloroplasts were visibly altered. In leaves of water-treated control plants, starch grains filled chloroplast stroma (Fig. 1A); these appeared similar to the CMNP treatment when observed 1 h after treatment (Fig. 1B). After 3 h, starch disappeared and plastoglobules were present (Fig. 1C). By 6 h after treatment, vesiculation and swelling were observed in thylakoid membranes, and the number of plastoglobules increased (Fig. 1D). Starch and soluble sugar content remained unchanged 1 h after CMNP treatment; however, after 3 h, starch declined and soluble sugars increased (Fig. 1E). Electrolyte leakage was unchanged until 3 h after CMNP application (Fig. 1F). By 24 h, a 60% increase in electrolyte leakage was measured in leaves treated with CMNP compared with the water-treated control. There was a marked loss of chlorophyll between 1 h and 2 h after CMNP application (Fig. 2A). Total leaf protein declined by 3 h after CMNP treatment and continued declining in a linear fashion, reaching 40% of the water-treated controls 12 h after treatment (Fig. 2B).

Transcriptional changes in response to CMNP

A summary of the functional distribution of genes up- and down-regulated by CMNP treatment is shown in Table 1, and Table 2 indicates expression of selected genes depicted in categories identified in Table 1 after 1, 3, and 6 h of CMNP treatment. These tables show genes up- or down-regulated by at least 2-fold. Genes associated with protein turnover/senescence, lipid signalling, detoxification, stress, and cell energetics/anoxia were up-regulated by CMNP treatment (Table 1). Several transcription/translation factors involved in regulation of various stresses and senescence responses were also induced. Increased treatment time increased expression of genes associated with detoxification and protein turnover/senescence. Transcripts down-regulated 1 h after CMNP application were associated with numerous metabolic areas. Increased treatment time increased the number of down-regulated genes related to sulphur metabolism and cellular structure. To confirm microarray results, nine marker genes for senescence, cell energetics, and stress were subjected to real-time PCR analysis (Table 3). Measured expression changes reflected changes determined in the arrays, demonstrating the fidelity of the microarray analysis.

Sucrose modified the response to CMNP

Control plants whose roots were treated with water showed no signs of wilting or stress (Fig. 3A, E). Visible leaf curl and dehydration occurred in plants exposed to CMNP (Fig. 3C, G). Addition of sucrose arrested this visible plant decline for 12 h (Fig. 3D, H); however, some wilting was observed after 24 h in the CMNP+sucrose treatment (data not shown). Sucrose alone promoted mild but transient leaf wilting 6 h after treatment (not shown), but plants appeared to recover after 12 h (Fig. 3B, F).

Discussion

This study integrates ultrastructural evidence with physiological and molecular data to examine the response of *A. thaliana* to CMNP, a pyrazole-derived plant growth regulator used as an abscission agent for citrus mature

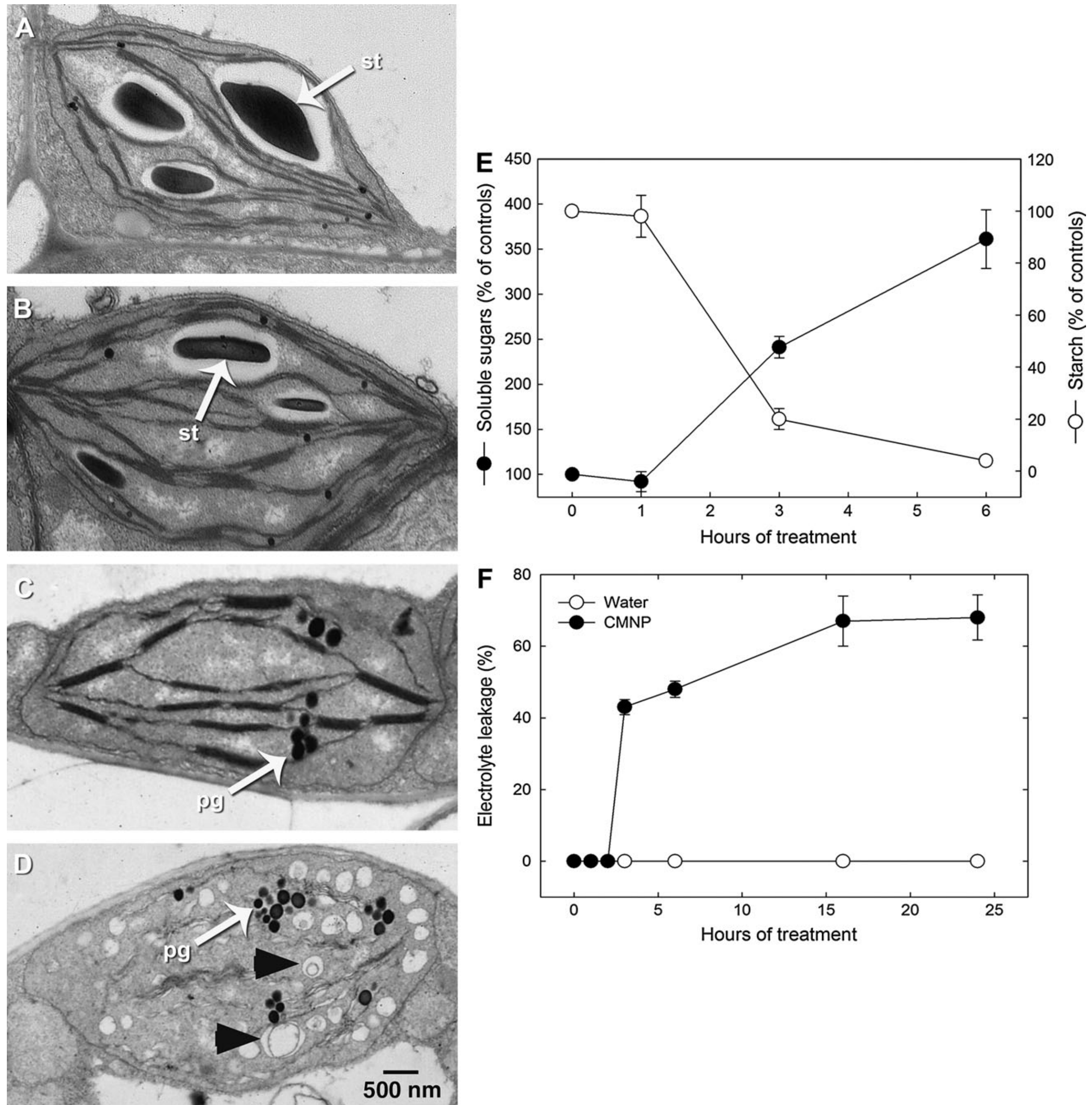

Fig. 1. Effect of 1.5 mM CMNP or water on *Arabidopsis* leaf chloroplast structure. Transmission electron microscopy pictures were taken in water-treated plants (A), or at 1 (B), 3 (C), or 6 h (D) after CMNP treatment. Arrowheads show thylakoid swelling and grana disorganization at 6 h. (E) Starch and soluble sugar content in *Arabidopsis* adult leaves treated with 1.5 mM CMNP over a 6 h period. (F) Electrolyte leakage in *Arabidopsis* adult leaves after treatment with 1.5 mM CMNP over a 24 h period. Pg, plastoglobules; st, starch.

fruit. Some of the responses to CMNP in citrus advancing abscission, maturation, and senescence were similar to those caused by ethylene, although different mechanisms seemed to operate (Alferez *et al*., 2005). Previous work with the ethylene perception inhibitor 1-methylcyclopropene showed that ethylene perception was not required for effective fruit loosening in citrus after CMNP or ethephon treatments, or peel wounding (Kostenyuk and Burns, 2004; Pozo *et al*., 2004). These results raised the possibility of both ethylene-dependent and ethylene-independent physiological mechanisms leading to mature fruit abscission in citrus, and suggested that CMNP's mode of action was, at least in part, independent of ethylene.

Root application of CMNP promoted electrolyte leakage, disrupted thylakoid membranes, and altered carbohydrate metabolism in *Arabidopsis* leaves. Symptoms of plant decline followed. Microarray analysis indicated that

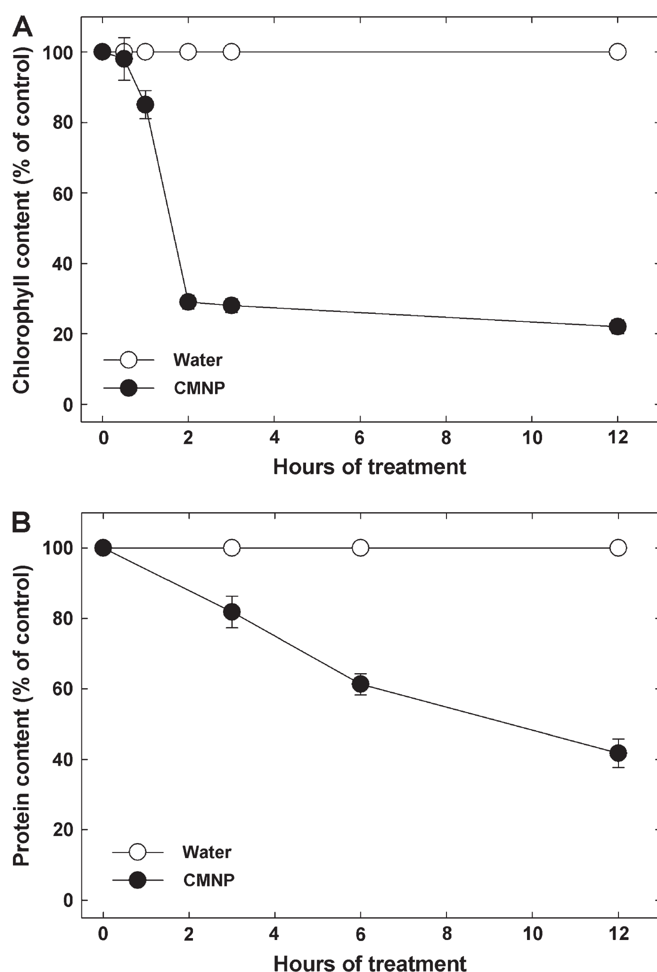

Fig. 2. Changes in total chlorophyll (A) and protein content (B) in *Arabidopsis* adult leaves after 1.5 mM CMNP or water treatment at various times up to 12 h continuous root exposure.

Table 1. *Functional distribution of up- and down-regulated genes in* Arabidopsis thaliana *adult plants 1, 3, and 6 h after treatment with 1.5 mM CMNP*

Numbers are the percentage of genes in each functional category. nd, none detected.

	1 h	3 h	6 h
Up-regulated genes			
Stress	23.5	13.3	15.4
Lipid signalling	8.8	16.7	7.7
Cell energetics/anoxia	26.5	13.3	7.7
Senescence/protein turnover	5.9	10	15.4
Transcription/translation factors	20.6	20	26.9
Detoxification	14.7	26.7	26.9
Down-regulated genes			
Stress	23.0	26.0	20.0
Cell energetics/anoxia	15.4	nd	7.0
Senescence/protein turnover	15.4	24.0	7.0
Transcription/translation factors	15.4	14.0	14.0
Detoxification	7.0	12.0	6.0
Cellular structure	7.7	12.0	20.0
Sulphur metabolism	nd	nd	20.0
Hormonal regulation	15.4	12.0	6.0

Table 2. *Fold change in up- and down-regulated genes of* Arabidopsis thaliana *leaves 1, 3, and 6 h after treatment with 1.5 mM CMNP*

Positive values indicate induction whereas negative values indicate repression. Expression changes of ⩾2-fold are listed.

Function/class	Time (h)			References
	1	3	6	
Up-regulated				
Stress				
Hsp70	4.80	2.49		Wu *et al*. (1988)
Chitinase	2.97		3.79	Cheong *et al*. (2002)
Putative endochitinase		2.23		Cheong *et al*. (2002)
Tyrosine aminotransferase		2.36	3.45	Lopukhina *et al*. (2001)
UDP-glucosyltransferase	3.10			Sullivan *et al*. (2001)
β-1,3-Glucanase	3.64			Wu and Bradford (2003)
Phosphatase 2C	9.89	2.54	3.88	Kreps *et al*. (2002)
Salt stress-induced tonoplast protein	5.60			Kreps *et al*. (2002)
NaCl-inducible protein			3.58	Kreps *et al*. (2002)
β-Xylosidase	5.90			Goujon *et al*. (2002)
Cellulose synthase	4.89			Joshi (2003)
CDPK-related protein kinase		6.29		Hrabak *et al*. (2003)
Lipid signalling				
OPR-1, 2	3.77	3.41	6.21	Schaller *et al*. (1998)
Patatin		3.28	3.44	Wang (2001)
Lipoxygenase	10.74			Porta and Rocha-Sosa (2002)
Lysophospholipase		4.20		Wang (2001)
Lipase/hydroxylase		2.76		Salzman *et al*. (2005)
Phospholipase D		3.16		Munnik *et al*. (2000)
Cell energetics				
Pyruvate dehydrogenase PDH1	2.38		3.80	Klok *et al*. (2002)
UDP-glucose dehydrogenase	3.85			Both *et al*. (2005)
Fructokinase FK1		2.91	3.57	Klok *et al*. (2002)
Phosphoenol pyruvate carboxykinase	3.08			Both *et al*. (2005)
Trehalose-P-synthase		3.38		Eastmond and Graham (2003)
Alcohol dehydrogenase ADH1	2.83	2.43		Dolferus *et al*. (2004)
Short chain ADH		3.52	5.33	Dolferus *et al*. (2004)
Pyruvate decarboxylase PDC1	2.84	2.10		Klok *et al*. (2002)
Malate oxidoreductase	3.57			Uyemura *et al*. (2004)
Photosystem I subunit	2.22			Hewezi *et al*. (2006)
ATPase I α subunit	2.53			Hewezi *et al*. (2006)
β-Amylase	8.60			Smith *et al*. (2005)
Senescence/protein turnover				
Glutamate dehydrogenase	5.41	2.13	5.54	Pageau *et al*. (2006)
Glutamine-dependent asparagine synthase		3.62	7.10	Hewezi *et al*. (2006)
3-Methylcrotonyl CoA carboxylase			2.89	Alban *et al*. (1993)
26S proteasome	2.13			Fu *et al*. (1999)
Carboxypeptidase Y-like protein			4.56	Bradley (1992)

Table 2. (*Continued*)

Function/class	Time (h)			References
	1	3	6	
Transcription/translation				
40S ribosomal protein	2.18			Klok *et al.* (2002)
60S ribosomal protein	2.25			Kim *et al.* (2004)
Spliceosomal-like protein	2.49			Ibrahim *et al.* (2001)
RING H2 zinc finger protein	2.39	3.41	2.62	Kreps *et al.* (2002)
Putative zinc finger protein	4.38		5.76	Kreps *et al.* (2002)
Trihelix DNA-binding protein	7.82			Smalle *et al.* (1998)
TGA 1 transcription factor DNA-binding protein	2.54			Lam and Lam (1995)
DNA-J-like protein		2.22	3.50	Randall and Crowell (1999)
MYB transcription factor			3.70	Kreps *et al.* (2002)
ATAF 1			3.33	Aida *et al.* (1997)
O-GlcNAc transferase		2.65	3.30	Izhaki *et al.* (2001)
DREB2A		2.98		Xiong *et al.* (2002)
MtN19-like protein		3.77	5.41	
WRKY DNA-binding protein		2.13		Mysore *et al.* (2002)
AP2 domain	2.60			Kreps *et al.* (2002)
Detoxification				
Cytochrome P450 monooxygenase	2.12	4.26	10.01	Siminzsky *et al.* (1999)
Stromal ascorbate peroxidase	2.20			Shigeoka *et al.* (2002)
ABC transporter	2.70	2.23		Kreuz *et al.* (1996)
Putative ABC transporter			4.29	Kreuz *et al.* (1996)
Glutathione *S*-transferase	3.02	2.05	7.79	Klok *et al.* (2002)
Putative glutathione *S*-transferase		2.95	5.64	Klok *et al.* (2002)
Heavy metal transporter	3.10			Hall (2002)
Nicotianamine synthase	17.20			Kim *et al.* (2005)
Serine acetyl transferase		2.48		Howarth *et al.* (2003)
Monooxygenase		2.33	3.80	Siminzsky *et al.* (1999)
1,4-Benzoquinone reductase		2.90	3.78	Matvienko *et al.* (2001)
Quinone oxidoreductase		2.90	4.24	Matvienko *et al.* (2001)
Down-regulated				
Stress				
Hevein-like protein	–3.46			Van Parijs *et al.* (1991)
Cytochrome P450	–2.74		–2.71	Siminzsky *et al.* (1999)
Cold acclimation protein			–2.52	Monroy *et al.* (1998)
12S storage protein		–2.37		Dodeman *et al.* (1998)
Stress-related protein	–2.52			Martinez and Chrispeels (2003)
Putative wound-induced protein	–2.71			Kreps *et al.* (2002)
Myrosinase-associated protein			–2.95	Taipalensuu *et al.* (1996)
Cell energetics				
Fructose 1,6-biphosphatase	–2.72			Klok *et al.* (2002)
Ribulose bisphosphate carboxylase (Rubisco small subunit)	–3.40			Sicher *et al.* (1981)
β-Amylase			–4.10	Lao *et al.* (1999)
Senescence/protein turnover				
19S Proteasome subunit			–3.11	Kwok *et al.* (1999)
Ketoconazole-resistant protein (sen-1)	–5.06			Schenk *et al.* (2005)
20S proteasome β-subunit	–3.20			Kwok *et al.* (1999)
Serine-threonine protein phosphatase		–2.37		Guo *et al.* (2004)
Putative peptide transporter		–2.40		Guo *et al.* (2004)
Transcription/translation				
APF 1		–1.89	–2.65	Hershko *et al.* (1980)
Putative transcription factor production of anthocyanin			–2.85	Montiel *et al.* (2004)
Non-phototropic hypocotyl 1	–2.04			Motchoulski and Liscum (1999)
Detoxification				
Peroxidase	–2.01		–2.05	Yamasaki *et al.* (1997)
Cellular structure				
Extensin-like protein			–2.28	Kieliszewski and Lamport (1994)
Pectinesterase-like protein		–2.92	–2.05	Koch and Nevins (1989)
Xyloglucan endo-transglycosylase-like protein			–1.87	Ma *et al.* (2004)
Subtilisin-like protein	–2.20			Neuteboom *et al.* (1999)
Sulphur metabolism				
Adenosine-5′-phosphosulphate-kinase (akn2)			–2.95	Schiffmann and Schwenn (1998)
Peptide methionine sulphoxide reductase			–2.57	Gustavsson *et al.* (2002)
S-Adenosyl-L-homocysteine hydrolase			–2.58	Weretilnyk *et al.* (2001)
Hormonal regulation				
GAST1 homologue			–3.11	Shi *et al.* (1992)
Putative auxin-regulated protein	–3.46			Zhao *et al.* (2003)
Cytokinin-repressed protein	–2.08			Toyama *et al.* (1999)

CMNP impacted several areas of metabolism involved in stress signalling. In general, the biological response to CMNP shared elements common to anoxia responses (Gibbs and Greenway, 2003), wounding (Pohnert, 2002), acceleration of abscission (Roberts *et al.*, 2002), and senescence (Buchanan-Wollaston *et al.*, 2003).

Numerous transcription/translation factors induced in response to various regimes of oxygen deprivation in

Table 3. *Quantitative real time-PCR (RT-PCR) analysis of nine representative senescence, cell energetics, and stress-related genes identified by microarray as being affected by CMNP*

Microarray data (m.a) are shown for comparison. For clarity, values with <2-fold induction and repression are also shown, although not included in Table 2. nd, not detected.

Category	Annotation	1 h		3 h		6 h	
		m.a	RT-PCR	m.a	RT-PCR	m.a	RT-PCR
Senescence							
GDH1	Glutamate dehydrogenase	5.4±0.4	3.3±0.4	2.1±0.3	1.6±0.2	5.5±0.1	3.3±0.3
Cell energetics							
RCO	Rubisco small subunit	–3.4±0.5	–2.5±0.2	–1.9±0.3	–2.0±0.1	–1.8±0.1	–2.1±0.3
ADH1	Alcohol dehydrogenase	2.8±0.4	4.0±0.2	2.4±0.9	5.5±0.3	1.9±0.6	2.0±0.1
PDC1	Pyruvate decarboxylase	2.8±0.3	4.0±0.1	2.1±0.1	3.9±0.3	nd	2.1±0.2
PDH1	Pyruvate dehydrogenase	2.4±0.2	1.1±0.3	nd	1.1±0.3	3.8±0.4	4.3±0.2
FK1	Fructokinase	1.9±0.4	2.0±0.1	2.9±0.1	2.2±0.2	3.6±0.4	4.8±0.4
TPS	Trehalose-P-synthase	nd	2.0±0.1	3.4±0.3	5.0±0.3	nd	2.1±0.1
Stress							
CHI	Chitinase	2.9±0.1	2.2±0.2	nd	1.0±0.2	3.8±0.5	2.7±0.3
GLU	β-1,3-Glucanase	3.6±0.3	2.9±0.1	1.12±0.2	1.0±0.3	1.9±0.4	2.0±0.3

Arabidopsis were also identified in this study, including heat shock factors, AP2 domains, zinc fingers, and WRKY factors (Klok *et al*., 2002; Branco-Price *et al*., 2005; Liu *et al*., 2005; Loreti *et al*., 2005). In addition, genes involved in early responses to abiotic stress (drought and cold, *DREB* genes; salt, oxidative stress enzymes, and temperature, MYB transcription factors; Kreps *et al*., 2002; Xiong *et al*., 2002) appeared to be similarly involved in the response of *Arabidopsis* to CMNP. Furthermore, expression of genes associated with detoxification increased with time, indicating plant responses leading to inactivation of CMNP. Together, these results indicate that CMNP induced stress-related metabolism.

Previous work demonstrated that CMNP treatment reduced total cellular ATP content, increased oxygen uptake in isolated mitochondria, and collapsed the electrochemical gradient in isolated chloroplasts (Alferez *et al*., 2005). Anoxic stress gene expression was influenced by CMNP, supporting the conclusion that the compound causes uncoupling. One consequence of uncoupling would be loss of stored reserves (Rawyler *et al*., 2002). Starch reserves were depleted, and swelling of thylakoid membranes occurred with CMNP treatment. Although thylakoid swelling is a symptom of several stresses (Barcelo and Poschenrieder, 1990; Anttonen, 1992; Holopainen *et al*., 1992), it has also been observed in mutants of *Chlamydomonas reinhardtii* that lack ATP synthase (Majeran *et al*., 2001). Swelling of thylakoid membranes in chloroplasts of *Arabidopsis* by CMNP treatment may be a result of direct inhibition of ATPase activity or inhibition of ATP synthesis via collapse of the electrochemical gradient.

Activation of detoxification mechanisms was evident after CMNP treatment. Despite increased induction of such genes as treatment time increased, plants were not able to overcome the toxic effects of CMNP. By 6 h, wilting was visible, starch content declined, and thylakoid membranes were disrupted. By contrast, in citrus fruit, stored reserves recover from canopy CMNP treatments, although fruit eventually abscise (Alferez *et al*., 2005, 2006). Continuous root uptake may explain the severity of the effect of CMNP in *Arabidopsis* as compared with citrus fruit where the compound is sprayed on the fruit surface, uptake is reduced, and/or rapid detoxification reactions inactivate CMNP.

CMNP treatment resulted in loss of starch in *Arabidopsis*. Carbohydrate shortage due to fruitlet-to-fruitlet competition and defoliation activates abscission in flowers and young fruitlets (Mehouachi *et al*., 1996). Loss of starch reserves and increased soluble sugars were features common to CMNP-treated mature citrus fruit (Alferez *et al*., 2006). Carbohydrate demand may activate carbohydrate metabolism to compensate for loss of reserves induced by CMNP treatment. Release of sugars and subsequent phosphorylation by hexokinases may also have a signalling function (Fox *et al*., 1998). Increased expression of *FK1*, *PDC1*, *ADH1*, and *TPS* genes following CMNP treatment suggests anoxic and/or sugar transcriptional regulation of downstream responses in both citrus and *Arabidopsis*, and support common effects in carbohydrate metabolism in both systems as a response to CMNP application.

In the presence of an uncoupler such as CMNP, electron transport from NADH to oxygen proceeds in a normal fashion, but ATP is not synthesized. Loss of respiratory control leads to increased oxygen consumption. In a situation of uncoupler-impaired respiration, pyruvate concentration increases and becomes available for fermentation (Tadege *et al*., 1999). Although pyruvate oxidation

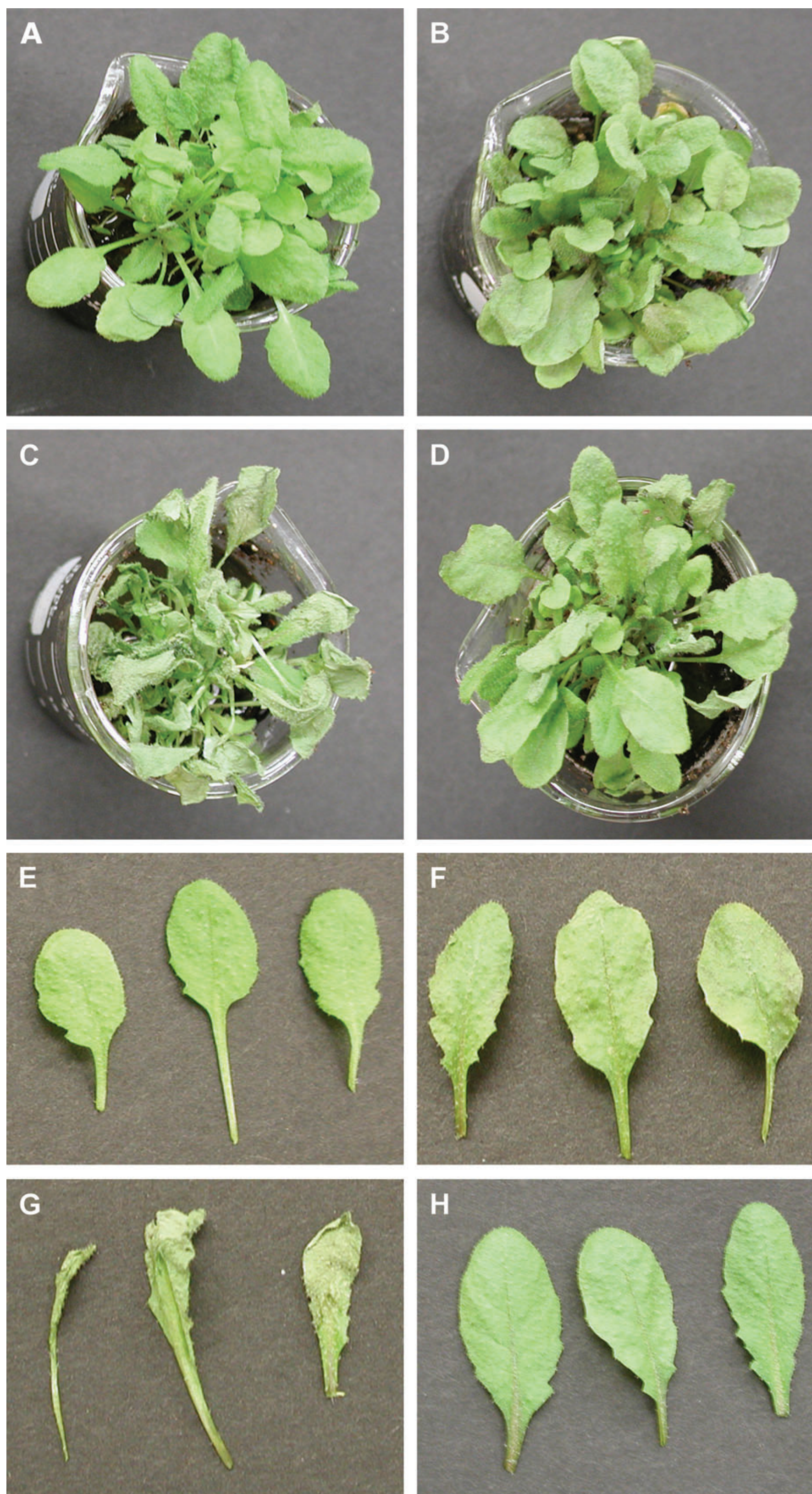

Fig. 3. Sucrose delays CMNP-induced wilting. *Arabidopsis* whole plants were treated with water, 1.5 mM CMNP, or 1.5 mM CMNP+2% sucrose. (A–D) Whole plants; (E–H) detached leaves. Images show results of one typical experiment after 12 h of treatment. A, E, water control; B, F, 2% sucrose; C, G, 1.5 mM CMNP; D, H, 2% sucrose+1.5 mM CMNP.

via the citric acid cycle will also occur, the lower K_m of PDH for its substrate ensures that enough pyruvate will be available for alcoholic fermentation via PDC1. Increased *PDC1* and *ADH1* expression has been associated with anoxia (Dolferus *et al*., 2004). However, once the carbohydrate pool was depleted, *PDC1* and *ADH1* expression decreased and plant decline could not be prevented. On the other hand, *FK1* expression increased as the carbohydrate pool depleted, probably due to increased respiration and general induction by anaerobic stress (Fox *et al*., 1998).

Anoxia triggers significant changes in gene expression, and exogenous sugar availability enhances tolerance to anoxia (Vartapetian and Jackson, 1997). Application of sucrose reduced CMNP-associated symptoms of plant decline and may have provided carbon substrate for carbohydrate metabolism in the absence of food reserves throughout the plant. Sucrose has been shown to allow extended accumulation of certain anoxia-induced transcripts such as *PDC* and *ADH* after stress (Loreti *et al*., 2005). After sucrose addition, plants remained healthy probably because continuous supply of sugar helped to overcome stress due to energy shortage.

Trehalose phosphate synthase (TPS) is an essential enzyme since disruption of AtTPS1 leads to an embryo-lethal phenotype in *Arabidopsis*. Evidence suggests that TPS is involved in regulation of sugar metabolism (Eastmond *et al*., 2003). Trehalose, whose first synthesis step is catalysed by TPS, may function as a stress metabolite (Goddijn *et al*., 1997; Eastmond *et al*., 2003). In the present study, *TPS* was induced by CMNP after starch disappeared in chloroplasts, strengthening the idea that CMNP disturbs sugar metabolism and supporting a role for trehalose as a storage metabolite and stress protectant.

Membrane breakdown in chloroplasts was indicated by disruption of thylakoids and increasing presence of plastoglobules with time after CMNP treatment (Fig. 1B, C). Plastoglobules have been associated with breakdown of photosynthetic membranes, chloroplast disorganization, protein degradation, and amino acid turnover during senescence (Guiamet *et al*., 1999). Furthermore, plastoglobules increase in number and size in senescing chloroplasts as thylakoid membranes degrade (Tuquet and Newman, 1980). Chlorophyll loss and protein degradation are also common features of senescence in green tissues (Buchanan-Wollaston *et al*., 2003). Catabolism of membrane lipids is accelerated during senescence, and free fatty acids are released. These membrane breakdown products can act as second messengers involved in signal transduction. Activation of the lipid signalling cascade after CMNP treatment was illustrated by visible disorganization of membrane structures in chloroplasts and up-regulation of genes coding for patatin-like proteins, PLA_2, phospholipase D (PLD), and 12-oxo-phytodiene reductase (OPR) before visual symptoms of membrane degradation. Additional evidence of membrane breakdown was shown by increased electrolyte leakage.

Sulphur assimilation and metabolism is crucial for plant survival during stress (Rausch and Wachter, 2005). Important compounds involved in xenobiotic detoxification, protein synthesis, and defence contain sulphur. The fact that sulphur metabolism-related genes were down-regulated by CMNP 6 h after treatment suggests that impaired detoxification and defence mechanisms could lead to rapid plant decline.

In conclusion, microarray and physiological data combined with ultrastructural observations in *Arabidopsis* show that root exposure to CMNP triggered signal cascades common to anoxia and senescence. The biological response to CMNP was characteristic of stresses associated with anoxia (Gibbs and Greenway, 2003), wounding (Pohnert 2002), acceleration of abscission (Roberts *et al*., 2002), and senescence (Buchanan-Wollaston *et al*., 2003). Lack of energy supply is a contributor to the final collapse of the functional cells, resulting in plant decline. The evidence presented indicated that exogenous sucrose applications provided short-term carbon necessary to maintain the plant, but could not overcome continuous CMNP exposure.

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