Detection of Stably Expressed Genes Contributing To PCD Triggered by Exogenous Oxalic Acid Treatment in Tobacco

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Abstract: The present study aims at detecting genes expressed at stable levels and contributing to PCD triggered by exogenous oxalic acid (OA) treatment (20 mM, pH 7.0) in tobacco (*Nicotiana benthamiana*). Qualitative and quantitative time-course analysis of cell death indicated some PCD-like features 24 hrs after OA treatment. Expression of a number of 17 genes contributing to PCD was also examined. Results of cell death in virus induced gene silencing (VIGS) corresponding to 14, out of the 17, genes indicated either increased or reduced amount of cell death. However, RT-PCR for the relative abundance of transcripts of these genes in tobacco wild type (WT) indicated that expression of six genes is not regulated at the transcriptional level. These genes are *MAPKα*, *NRC1*, *WIPK*, *RAR1*, *SIPK* and *FLS2*. The results of VIGS corresponding to five, out of the six, genes indicated a reduced relative amount of cell death under OA treatment for 24 hrs as compared to the WT. Only one VIGS corresponding to *FLS2* gene resulted in an increased amount of cell death under OA treatment for 24 hrs. This indicates that OA triggers PCD, but has no influence on triggering these six PCD-related genes. Besides, these six genes might be regulated at step(s) downstream transcription. Our results will scope the light on the possible use of these genes in conferring resistance to environmental stimuli by retarding the machinery of PCD in plant.

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1. Introduction

Apoptosis or PCD is a highly conserved process that is used to remove unwanted cells in eukaryotes. As in animals, a programmed type of cell death occurs in plants as part of normal growth and development, including reproduction, seed germination, aerenchyma formation, tracheary element differentiation, and senescence (Filonova *et al.,* 2002, Kuriyama and Fukuda, 2002, Lim *et al.,* 2007, Turner *et al.,* 2007) in which initiation phase involves signaling cascades that prepare the cell for entry into the execution phase. PCD in plants is less studied compared with animals, but it is well known to be triggered by biotic and abiotic stresses (Zhang and Klessig 2001). Although release of mitochondrial cytochrome c has been identified in plants (Thomas and Franklin-Tong 2004, Yao *et al.,* 2004, van Doorn and Woltering, 2005), many genes, encoding components of the apoptotic machinery, either have not yet been identified in plants or are simply not present (Woltering *et al.,* 2002).

Emerging evidence has indicated, in necrotrophic fungus–plant interactions, active involvement of the pathogen in influencing host pathways to direct plant cells towards death (Van Baarlen *et al.,* 2004). For example, *Sclerotinia* sp. secretes a wide array of substances to facilitate their necrotrophic life style within the plant cell (Annis & Goodwin, 1997). In addition to cell-wall-degrading enzymes, oxalic acid (OA) plays a key role in pathogenesis, fungal development and plant cell death (Dutton and Evans, 1996). OA is toxic, non-specific phytotoxin, which degrades or weakens the plant cell wall via acidity. It was noted that gene expression of mitogen-activated protein kinases (MAPKs), required for sclerotial formation in *S. sclerotiorum*, is activated by acidic pH mediated via OA (Rollins and Dickman, 2001, Chen *et al.,* 2004, Kim *et al.,* 2008).

Virus-induced gene silencing (VIGS) is a method that takes advantage of the plant RNAi-mediated antiviral defense mechanism (Velásquez *et al.,*2009). With virus vectors carrying sequences

derived from host genes, the process can be targeted against the corresponding host mRNAs. VIGS has been adapted for high-throughput functional genomics in plant by using the Ti plasmid of the plant pathogen *Agrobacterium tumefaciens* to deliver a recombinant virus carrying part of the gene sequence targeted for silencing. Then, systemic virus spread and the plant's endogenous RNAi machinery accelerate the silencing process in which dsRNAs corresponding to the target gene are produced and then cleaved by the ribonuclease Dicer into siRNAs of 21-24 nucleotides in length. These siRNAs ultimately guide the RNA-induced silencing complex (RISC) to degrade the target transcript (Carrington & Ambros 2003). Different vectors have been employed in VIGS and one of the most frequently used is based on tobacco rattle virus (TRV). Two different *A. tumefaciens* strains are used for VIGS, one carries pTRV1, which encodes the replication and movement viral functions, while the other carries pTRV2, which harbors the coat protein and the sequence used for VIGS (MacFarlane, 1999, Liu *et al.,* 2002). Inoculation of *Nicotiana benthamiana* seedlings with a mixture of both strains results in gene silencing. Silencing of the endogenous phytoene desaturase (*PDS*) gene, which causes photo-bleaching, is used as a control for VIGS efficiency. RNA transcript abundance of the gene of interest is measured to ensure that it has efficiently been knocked down (Senthil-Kumar *et al.,* 2007).

In this report, we show that exogenous OA functions as an elicitor of PCD or apoptotic-like features in tobacco plants by triggering pathways and signals responsible for PCD. A number of candidate genes related to PCD will be utilized. Some of which are known to function during plant's defense against invading pathogens via a mechanism known as the hypersensitive response (HR) (Ren *et al.,* 2006).

2. Materials and Methods

Plant material and bacterial strains

Wild-type tobacco (*Nicotiana benthamiana*) plants were grown from seed in a growth room at 25°C with 16-hrs light periods as previously described (Dickman *et al.,* 2001). *Agrobacterium tumefaciens* and *Escherichia coli* strains were grown in Luria-Bertani medium at 30 and 37°C, respectively. Antibiotics were used at the following concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml) and rifampicin (100 µg/ml).

Virus induced gene silencing (VIGS)

VIGS of a number of PCD-related genes (Table 1) were kindly provided by Professor GB Martin, Professor of Plant Pathology and Plant-Microbe Biology, Boyce Schulze Downey Research Chair, Boyce Thompson Institute, Cornell University, Tower Road, Ithaca, NY 14853-1801, USA. VIGS were utilized as described by Velásquez *et al.,* (2009). All *N. benthamiana* plants were kept in a growth chamber with a 16-h day length, a temperature of 20-22°C, and 50% relative humidity (RH) for 25 days before they were used for the assays. *A. tumefaciens* harboring pTRV2, pQ11, pTRV2::PDS and pTRV2: or Q11::target gene fragment are grown at 30°C for 2 days on LB agar plates supplemented with 50 µg/ml of kanamycin and 100 µg/ml of rifampicin. The kanamycin selects for the pTRV2 and pQ11 plasmids, while the rifampicin for the *Agrobacterium*. Silencing of PDS causes the plants to photo-bleach and is used as a control to detect silencing efficiency. Gateway compatible pTRV2 vector was used by the provider for cloning as described by Liu *et al.,* (2002). For the PDS-silenced control plants, photo-bleaching starts to be seen in new leaves 10 days after infiltration up to senescence.

Table 1. List of VIGS utilized in the present work to detect knock down of a number of genes contributing to PCD triggered by oxalic acid. pTRV2 and pQ11 are empty vectors, with different antibiotic resistance genes, used as negative controls. pQ11:PDS is the VIGS causing photo-bleaching (positive control) in new leaves when *PDS* gene is knocked down.

Stock no.	Construct	Reference
3698	pTRV2	(Velásquez *et al.,* 2009)
3835	pQ11	
3836	pQ11::PDS	(Velásquez *et al.,* 2009)
3861	pQ11::NbMAPKα	(del Pozo *et al.,* 2004)
4850	pTRV2::NRC1	(Gabriëls *et al.,* 2007)
3594	pTRV2::WIPK	(Ekengren *et al.,* 2003)
3582	pTRV2::RAR1	(Ekengren *et al.,* 2003)
3589	pTRV2::SIPK	(Ekengren *et al.,* 2003)
4892	pQ11::FLS2	(Ekengren *et al.,* 2003)

OA treatment and measurement of cell death

Leaf discs of different WT and VIGS of *N. benthamiana* plants were obtained from 7-week-old plants using a 10-mm-diameter cork borer. Then, discs were submerged in OA (Sigma-Aldrich) at 20 mM for 24 hours. Cell death was determined and quantified by using Evans blue as described (Kim *et al.,* 2008). Cell membranes are permeable and, thus, stained when cell death occurs (Baker and Mock, 1994). Briefly, treated leaf discs were submerged in 0.25% (w/v) Evans blue solution (MP Biomedicals, Santa Anna, CA, USA) for 20 min. Excess and unbound dye was removed with deionized water. The discs were placed in liquid nitrogen, homogenized, placed in 1% (w/v) sodium dodecyl sulfate (SDS) solution, and incubated for 10 min at 37°C. Solutions were centrifuged at 13,000 ×*g* for 5 min. The quantity

of remaining dye was spectrophotometrically measured at 600 nm. Measurements were expressed as relative values, with 1 corresponding to a maximum of a sample tested.

For the detection of DNA laddering as a sign of PCD, total plant DNA was extracted from leaf discs using a modified method (Ryerson and Heath, 1996). Discs were homogenized using liquid nitrogen, incubated for 30 min at room temperature in DNA extraction buffer (0.1 M glycine, 50 mM NaCl, 10 mM EDTA, 2% SDS and 1% sodium lauryl sarcosine) and mixed with an equal volume of Tris-saturated phenol. The mixture was centrifuged for 15 min at 10,000 ×*g*. The supernatant was treated with chloroform/isoamyl alcohol (24:1, v:v). After re-centrifugation at 10,000 ×*g*, DNA was precipitated with a twofold volume of 100% ethanol, washed with 70% ethanol and dissolved in Tris-EDTA buffer containing RN*ase* A (40 µg/ml). DNA was recovered after phenol extraction and ethanol precipitation. DNA samples were separated on a 1.5% agarose gel in 0.5x Tris-borate-EDTA, stained with ethidium bromide and visualized using the Gel Doc XR from Bio-Rad.

RT-PCR to analyze gene expression

Leaf discs of 7-week-old *N. benthamiana* plants were submerged in OA (Sigma-Aldrich) at 20 mM for 3, 6, 12 and 24 hours. Then, discs were frozen in liquid nitrogen to extract RNAs. Untreated leaf discs were used as a negative control. Total RNA was extracted from three similar-sized (10 mm^2) leaf discs per plant (approximately 50 mg tissue) collected from upper leaves using Trizol (Invitrogen) and treated with RNase-free DNase (Promega). First-strand cDNA was synthesized using 2.5 ug of total RNA, 0.5 ug oligo (dT) primer and Superscript II reverse transcriptase (Invitrogen) to a final volume of 20 ul. PCR was performed in 20-ul reactions using 1 ul cDNA, 1x PCR buffer (with 1.5 mM $MgCl_2$), 200 uM dNTPs, 200 nM of each gene-specific primer and 0.2 U of Taq DNA polymerase (Promega). To ensure that no DNA contamination occurred, PCR for the original RNA samples was run and results were negative (Data provided upon request). To ensure that similar amounts of cDNAs were used for silenced and non-silenced genes, parallel reactions with *actin* primers were run as a positive control. Each PCR cycle included denaturation at 94°C for 15 sec, annealing at 48-52°C for 30 sec, and extension at 72°C for 45 sec. Number of cycles was 40 for amplifying fragments of all genes except *RAR1* whose PCR was run for 50 cycles. Amplicons were analyzed on a 1.2% agarose gel stained with ethidium bromide and visualized using the Gel Doc XR from Bio-Rad.

RT-PCR was performed twice. The first was done for RNAs of WT plant discs to detect transcript levels of all genes (Table 1) across time of OA treatment. The second was done for VIGS of each gene untreated with OA to prove knock down of corresponding gene (data provided upon request). RT-PCRs were done for replicated samples with similar results. Also, the two RT-PCRs were repeated with similar results.

3. Results and Discussion

Effectiveness of VIGS in silencing target genes in tobacco

To examine the effectiveness of VIGS in tobacco, pTRV2 carrying a fragment of the phytoene desaturase gene, TRV2::PDS, was used in silencing. The results indicated that silencing of the *PDS* gene inhibited carotenoid biosynthesis and gave rise to a visible phenotype of photo-bleaching of the new leaves (Figure 1). Photo-bleaching of leaves was uniform throughout the plant in which none of the leaves turned completely white. This indicates the homogeneous low rate spread of the virus and partial silencing of the gene in the plant. This phenomenon can be advantageous when silencing PCD-related genes.

Figure 1. Silencing of the *PDS* control gene causes photo-bleaching in *N. benthamiana* plants. Photographs were taken 21 days after infiltration.

Based on these results, we concluded that TRV-based silencing is potentially effective for characterizing the possible involvement of OA in triggering apoptosis in PCD-related genes. VIGS has previously proven useful for a detailed characterization of the role of PCD-related MAPKs (mitogen-activated protein kinases) cascade such as MAPKKKα (del Pozo *et al.,* 2004). One of which, namely NPK1, plays a role in the placement of certain MAPKs, or calcium-dependent protein kinases (CDPKs), in the N and Cf9 pathways (Romeis *et al.,* 2001, Jin *et al.,* 2002, 2003). VIGS occurs naturally in plants when they are infected with a virus carrying sequences homologous to a host nuclear gene. The virus infection triggers the cytoplasmic degradation of any RNA with sufficient homology to the target sequence via RNAi machinery

(Lindbo & Dougherty 1992, Ding 2000, Fagard *et al.,* 2000, Lindbo *et al.,* 2001, Vance & Vaucheret, 2001). This activity results in post-transcriptional silencing (PTGS) of the homologous nuclear genes, and resistance to viruses containing the target sequence. Many studies have demonstrated that VIGS is a powerful tool for systematically shuttling plant gene sequences into plants and screening infected plants to determine gene function (Lindbo *et al.,* 2001, Vance and Vaucheret, 2001, Sahu *et al.,* 2012).

Apoptosis-like cell death in tobacco cells induced by OA

Qualitative and quantitative time-course analysis of cell death upon treatment with OA in tobacco WT leaf discs were measured using Evans blue staining. Gradual cell death was clearly observed across time of OA treatment (Figure 2a&b). Our results indicated that treatment beyond 24 hrs is not necessary. Longer treatment time (48 hrs) resulted in recovering poor quality RNAs for further gene expression analysis. Kim *et al.,* (2008) indicated substantial increases in tobacco cell death 48 through 96 hrs after OA treatment with concentration (20 mM) similar to this in the present study. However, they indicated that 96 hrs treatment resulted in extensive tissue collapse. This reflects the difficulty of isolating good quality RNA for gene expression analysis. They also indicated that lower concentration of OA (10 mM) yielded similar but delayed results. Based on the kinetics of the cell death in response to OA, tobacco DNA was isolated at several time points. DNA cleavage resulting in a characteristic DNA laddering pattern associated with apoptotic cell death started to appear 24 h after OA treatment (Figure 2b). Kim *et al.,* (2008) proved that PCD induction is not due to the acidic nature of OA but rather to a property of OA itself.

PCD is a major process by which eukaryotes regulate normal growth and development as well as stress responses (Vaux and Strasser, 1996). Accumulating evidence shows that inappropriate regulation of PCD can have inadequate consequences for a given organism. In humans, cancer can be promoted when PCD does not occur, while it normally occurs. Cell death has been correlated with the accumulation of ROS during the oxidative burst (Dickman and Reed, 2003). OA was reported by Kim *et al.,* (2008) as a non-specific phytotoxin in plant tissue. Rather, it functions as a signaling molecule or elicitor to subvert and redirect host pathways toward cell death with the involvement of ROS. The latter are major components induced under oxidative stresses such as H_2O_2 and hallmarks of apoptotic-like cell death. Kim *et al.,*(2008) also indicated that OA is multifunctional as it could lower the pH, hence, enhance fungal pathogenicity and induce PCD by inhibiting the oxidative burst of the plant.

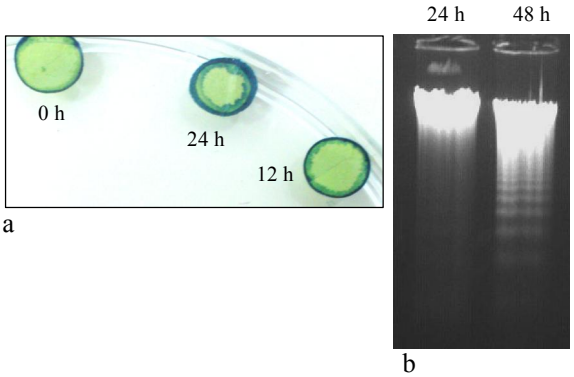

Figure 2. OA-induced cell death as measured by Evans blue staining (a) and DNA laddering (b) in tobacco leaf discs across time of treatment (0, 12 and 24 hrs for staining experiment and 24 and 48 hrs for DNA laddering experiment).

Several evidences were consistent with OA being a specific elicitor of plant PCD. First, OA induces DNA laddering in a time- and dose-dependent manner (Kim *et al.,* 2008). Second, OA-deficient nonpathogenic mutant culture filtrates of fungus did not exhibit laddering, however, laddering was restored when OA was exogenously supplied. Third, other organic acids at similar concentrations were unable to induce ladders. Forth, OA-induced PCD does not occur at acidic pH (3 to 4), but rather at pH 5 and 6. Low pH can cause cell death but it does not appear to be programmed, thus, represents an OA-producing pathogenic fungal necrotic type of death.

Detection of genes contributing to PCD triggered by OA

Many genes with a role in PCD-like features were detected in tobacco. Seventeen of these genes were utilized in the present study. Results of cell death in VIGS corresponding to these genes indicated that silencing 14 of them either increased or reduced amount of cell death (data provided upon request). The other three VIGS include MEK1, cyclophilin and cytochrome C. The mean relative cell death for these three VIGS after 24 hrs of OA treatment was about 0.76, which is the mean value for the WT plant (Table 2). However, RT-PCR for the relative abundance of transcripts of these genes in tobacco WT indicated that expression of six, out of the 14, genes was unchanged across time of OA treatment (Figure 3). This indicates that these genes are not regulated by OA treatment at the transcriptional level. Similarly, the MAPK cascade of genes was shown to be regulated by various mechanisms, including transcriptional, translational regulation and post-

transcriptional regulation such as protein-protein interactions (Katuo *et al.,* 2005, Taj *et al.,* 2010). In the present study, the results of VIGS corresponding to five, out of the six, genes indicated a reduced relative amount of cell death under OA treatment for 24 hrs as compared to the WT (Table 2). Only one VIGS corresponding to *FLS2* gene resulted in an increased amount of cell death under OA treatment for 24 h. The results of RT-PCR for VIGS corresponding to these six genes untreated with OA resulted in the production of no amplicons. These results indicate the silencing of these genes in VIGS (data provided upon request).

Table 2. Multiple comparisons of the mean relative cell death as responses of tobacco WT and VIGS leaf discs following OA (20 mM, pH 7.0) treatment for 24 h as determined by Evans blue staining. Dye released from dead cells was measured at absorbance at 600 nm. Measurements were expressed as relative values with 1 corresponding to the maximum of a sample. Data are presented as means from two independent experiments with three replicates each.

VIGS	Treatment for 24 hrs		Response
	Oxalic acid	Water	
WT	0.75^{B}	0.33^{A}	Control
pTRV2	0.76^{B}	0.26^{A}	Control
MAP3Kα	0.56^{C}	0.30^{A}	Reduced amount of cell death
NRC1	0.50^{C}	0.28^{A}	Reduced amount of cell death
WIPK	0.62^{C}	0.33^{A}	Reduced amount of cell death
RAR1	0.63^{C}	0.31^{A}	Reduced amount of cell death
SIPK	0.54^{C}	0.29^{A}	Reduced amount of cell death
FLS2	1.00^{A}	0.31^{A}	Increased amount of cell death

Means within each column followed by the same letter are not significantly different by Duncan's New Multiple Range test (<0.05).

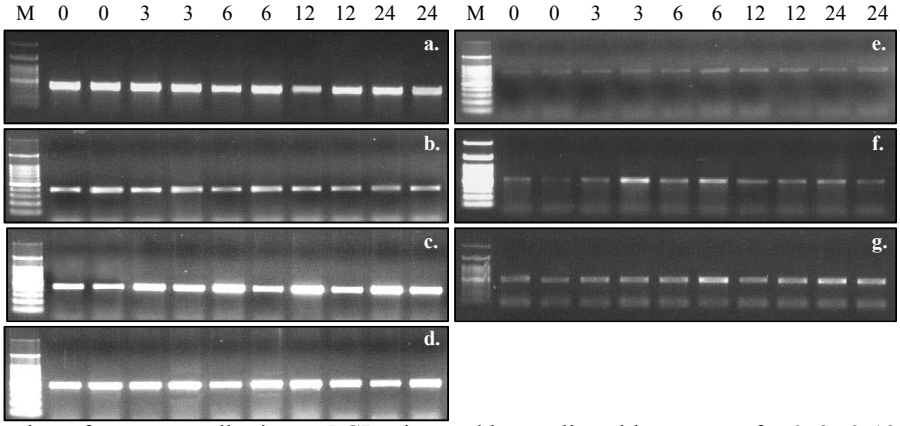

Figure 3. Expression of genes contributing to PCD triggered by oxalic acid treatment for 0, 3, 6, 12 and 24 h with unchanged rate of expression in tobacco wild type plants. a. *actin* (positive control), b. *MAPKα*, c. *NRC1*, d. *WIPK*, e. *RAR1*, f. *SIPK*, g. *FLS2*.

MAPKs are highly conserved across eukaryotes. They are Ser/Thr kinases that are activated by dual phosphorylation of Thr and Tyr residues in a TXY motif. In plants, MAPK cascades have emerged as key players in some of the most essential roles in plant signaling networks. They have been shown to be activated by a variety of stresses, including pathogen infection, wounding, drought, salinity, osmolarity, UV irradiation, ozone, and reactive oxygen species (Tena *et al.,* 2001, Zhang and Klessig, 2001, Li *et al.,* 2007). Activation can involve types of regulation other than transcriptional regulation. Some of which, ex. Arabidopsis AtMPK3, are involved in osmotic and oxidative stresses, as well as in abscisic acid and pathogen elicitor signaling (Nakagami *et al.,* 2005). Other genes like *NRC1* (NB-LRR protein required for hypersensitive response (HR)-associated cell death 1) are also associated with cell death (Gabriëls *et al.,* 2007). The gene is involved in multiple HR and resistance pathways. Knock down of *NRC1* in tomato confirmed its requirement for induced HR, and revealed that the gene is also required for Cf-4-mediated resistance to *C. fulvum*. MAPKKKα gene was reported to be involved in regulation of host cell death during both immune and susceptible plant-pathogen interactions (del Pozo *et al.,* 2004). The latter authors presented evidence that the signal transduction pathway

initiated by MAPKKKα involves two distinct MAPK cascades, the first is well-known to result in HR cell death via salicylic acid–induced protein kinase (SIPK) regulation and the other is speculated to result in disease-associated cell death (Figure 7, del Pozo *et al.,* 2004). SIPK and wound-induced protein kinase (WIPK) are two tobacco (*Nicotiana tabacum*) MAPKs that are rapidly activated after various challenges, such as osmotic stress (Droillard *et al.,* 2000; Mikolajczyk *et al.,* 2000) and are activated after wounding. Using transgenic cultivated tobacco, Wu *et al.,* (2007) demonstrated that both SIPK and WIPK regulate each other's transcript accumulation, highlighting the complicated transcriptional crosstalk that occurs among protein kinases. Multiple genetic screens have led to the identification of RAR1 (Required for MLA12 Resistance 1) inducer as a key component required for several R protein functions. RAR1 has been shown to be co-chaperons for the HSP90 (Heat-Shock Protein 90) protein responsible for the stability of some R proteins (Shirasu, 2009). This gene was reported to be located upstream the cascade of events towards disease resistance through the downstream MAPKKs, MEK1 and MEK2 and the MAPKs, WIPK and Ntf4 during Pto-mediated disease resistance pathway in tomato (Ekengren *et al.,* 2003, Ren *et al.,* 2006). FLS2 is a pattern recognition receptors (PRR, Chakravarthy *et al.*, 2010) recognizing pathogen-associated molecular patterns (PAMPs) to trigger PAMPs-triggered immunity (PTI) (Bent and Mackey, 2007). Plants with disrupted expression of the *FLS2* gene are compromised for PTI and more susceptible to certain bacterial pathogens (Heese *et al.,* 2007).

In plants, MAPK signaling appears to involve cross-talk with a variety of stress responses forming complex interconnected networks within cells (Zhang and Klessig, 2001, Asai *et al.,* 2002, Pedley & Martin, 2005). Tobacco NtMEK2 (MAP2K)-SIPK/WIPK are reported to be involved in osmotic stress (Kiegerl *et al.,* 2000, Yang *et al.,* 2001, Kroj *et al.,* 2003). In the present study, we have detected six PCD-related genes are not regulated at the transcriptional level. However, they were found to trigger PCD as indicated by the Evans blue staining of dead cells. This indicates that OA triggers PCD, but has no influence on triggering these PCD-related genes. We conclude that these genes might be regulated at step(s) downstream transcription by binding encoded protein to other proteins, protein phosphorylation, activation by SA or other transcription factors, etc. Further investigation is required in order to detect the proper level of regulation of these PCD-related genes. This will scope the light on the possible use of these genes in conferring resistance to environmental stimuli or in retarding the machinery of PCD in plant.

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