Project Title: **RESISTANCE TO PIERCE'S DISEASE BY TRANSGENIC EXPRESSION OF PLANT-DERIVED ANTI-APOPTOTIC GENES**

Contract Number: SA7220
Time period covered by the progress report: November 1, 2007 to March 1, 2008

Principle Investigators: David Gilchrist (dggilchrist@ucdavis.edu); (530)-304-6007
James Lincoln (jelincoln@ucdavis.edu) (530)752-6587
Dept. of Plant Pathology, Univ. of California, Davis, CA 95616.

List of objectives, and description of activities conducted to accomplish each objective

Objectives:

1. Produce transgenic grape plants over-expressing candidate anti-apoptotic plant genes obtained through conditional life-death cDNA library screens.
2. Continue to evaluate recently obtained Thompson Seedless transgenic grape plants expressing the 6 candidate anti-apoptotic genes for blocking of PD symptoms.
3. Measure the effect of blocking PD symptoms with anti-apoptotic transgenes on *X. fastidiosa* (*Xf)* bacterial population levels and movement in the xylem by quantitative PCR (qPCR) and confocal laser scanning fluorescence microscopy to monitor GFP-tagged *Xf.*
4. Graft resistant transgenic rootstocks of VvPR1 and cDNA 456 to untransformed scions of Thompson Seedless to monitor movement of either expressed proteins of these genes that contain a secretory leader on VvPR1 or the RNA derived from the 3'UTR from the ortholog of the potato p23 gene that shares stem and loop homology to the 3'UTR of Bcl2. **New Objective (added due to results in the past 6 months)**.
5. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape
6. Determine gene expression changes in transgenic grape compared with non-transgenic plants infected with *X. fastidiosa*.

Activities conducted to Accomplish Objectives including objectives added recently

- **Produce grape transgenic plants over-expressing candidate anti-apoptotic plant genes obtained through cDNA library screens**.

  We now have 10-20 transgenic plants of each construct listed in Table 1. Clonal copies of these plants were inoculated with *Xf* at the end of January 2008 (Figure 1). The cv. Freedom, a common rootstock, is highly susceptible to PD and we, along with Dr. Lindow independently, demonstrated that Freedom responds to PD with the same symptoms as Chardonnay and Thompson Seedless (10,11). VvPR1 and 456 3'UTR transgenics will be used test for the movement across a graft union as indicated in objective 4.

- **Evaluate effect of specific anti-apoptotic plant genes in grape on *Xf* and PD symptoms *in planta*,** *Xf*- GFP movement and relative concentration has been determined by quantitative PCR (qPCR) and confirmed with confocal laser scanning fluorescence microscopy of

individual stems of side branches. The data is collected on a time course basis following *Xf*-inoculations using the needle puncture of the stem method to allow uptake of 10-20 µl of *Xf* at $2x10^8$ cfu/ml. Additional inoculations are in progress using serial dilutions of *Xf* to quantify the effect of inoculum concentration on symptom suppression in transformed and untransformed plants to assess the effectiveness of the anti-PCD genes at initial inoculum levels more likely to occur under vineyard conditions with natural GWSS inoculation. Ultimately, we anticipate using GWSS inoculation.

- **Grafting experiments:** We have successfully grafted cv. Thompson Seedless onto Freedom rootstocks transgenic for VvPR1 and cDNA 456 to initiate preliminary experiments on the ability of the protein of VvPR1 and the RNA of cDNA 456 to cross the graft union. If either are detected in the Thompson Seedless scion, inoculation experiments of the scion will be undertaken. We have just developed antibodies against PR1 and have a PR1-GFP fusion construct, which has been validated to express GFP in the apoplast around cells induced to die by PCD. The 456 RNA will be detected by PCR, primers for which have been validated in our lab. We also will test the conserved stem-loop sequence isolated from the rest of the 456 UTR for ability to block PD symptoms and to move across a graft union.

.

- **Determine grape gene expression changes in transgenic compared with non-transgenic plants infected with *Xf*.** Doug Cook previously reported extensive changes in gene expression in *Xf*- infected Cabernet Sauvignon on Freedom rootstock by Real-Time PCR followed by expression profiling (9). We will assess the effect of the expression of the anti-PCD genes on the 24 genes he reported to be up-regulated only in infected tissue by Real-Time PCR. We also will assess which genetic pathway is affected by the transgenes by difference in expression profiling of inoculated transgenic and non transgenic cohorts.

Research accomplishments and results for each objective

Overall summary statement:
In the past 6 months we successfully demonstrated resistance against PD symptoms in the susceptible grape rootstock cv. Freedom by transgene expression of PCD suppressive endogenous grape genes VvPR1, CB390 (metallothionein) and a translation-associated regulatory 3'UTR sequence, named cDNA 456, afford protection against symptoms and death of grape plants infected with *Xf* (Figure 4). (See discussion page 4). All three cDNAs were recovered anonymously from the plant-based cDNA screen and all have functional links with conserved domains to anti-apoptotic orthologs in the animal kingdom (1, 17, 18). In addition, these genes have the potential of systemic delivery of proteins or RNA regulatory sequences across a graft union as will be discussed later. Hence, grafting experiments involving transgenic Freedom root stocks of VvPR1 and cDNA 456 to untransformed Thompson Seedless FPS 02A were initiated recently as an additional objective #4

- We further demonstrated that expression of these three sequences, not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at 10^4 bacterial cells per gram of stem tissue, which is 4 orders of magnitude below the level observed in untransformed plants that died within 2 months (10^8 bacteria per gram of stem tissue) following controlled inoculations in the greenhouse (Table 3, Figure 4). The key point here is that altered expression of the anti-apoptotic transgenes does not kill the bacteria but

does restrain the titer in the asymptomatic transgenic plants from a lethal level of 10^8 to a level of 10^4 cells/gm stem tissue in the most resistant lines; the 10^4 titer is equivalent to that found in the asymptomatic host *V. californica* 12 months after inoculation.

- Interestingly, the 10^4 cells/gm stem tissue titer level in the asymptomatic transgenic plants and *V. californica* is the same observed by Dr. Lindow in his *rpfF* transformed plants that also are asymptomatic suggesting that susceptible grape plants can tolerate a bacterial population at 10^4 cells/gm without showing PD symptoms. In both situations, PD symptoms are suppressed but low levels of bacteria remain in the plants at levels equivalent to those found in the asymptomatic host *Vitis californica*.
- These data confirm the role of PCD in Pierce's Disease and illustrate a genetic mechanism using altered expression of endogenous grape genes to achieve sustainable resistance by suppressing the response of susceptible grapes to *Xf*, while coincidentally reducing the titer of the bacteria in the xylem.
- Ongoing experiments will further define and characterize a mechanism to suppress PD in cultivated grapes without eliminating the bacteria from the plant. The experiments with the genes listed in table 2 that have now been re-introduced into the *V. vinifiera* cv Thompson Seedless under control of a constitutive promoter. The first set of these plants were inoculated with *Xf* in January 2008. The assays will determine disease suppression, the behavior, movement and titre of the bacteria in relation to sites and timing of inoculation.
- Grafting experiments: These have been initiated with CB456 and VvPR1, the latter which has a signal sequences for extra cellular secretion and the PR1 translated protein has been isolated from expressed plant sap. This indicates the potential for systemic movement and function distal to the site of secretion for PR1 and the putative microRNA of CB456 also has potential for systemic movement as indicated earlier

Specific comments related to VvPR1 and cDNA 456 as fundamental discoveries with global implications to diseases in addition of PD, but with direct relevance to PD.

VvPR1: PR1 is widely used as a marker for resistance, including the onset of systemic acquired resistance (SAR) but has no known function, although the gene was first identified more than 2 decades ago. Sequence comparison of VvPR1 within genome databases using the Clustal W algorithm confirmed high conservation of several domains in orthologs of the gene analyzed from humans, dog hookworm, *Meloidogyne incognita*, grape, tomato and alfalfa. In each case where expression is reported to be induced is a situation in which apoptosis is blocked or suppressed. Specifically, in the case of plant response to disease, the presence of the PR1 message occurs in situations in which the induction of PCD during the compatible infection process is a dependent step and where blocking death, chemically or genetically provides resistance. Localized expression of the protein at the point of PCD induction is consistent with the suppression of pathogen growth and confinement to a zone surrounding the lesion, a situation that occurs in this and other plant-bacterial interactions as pointed out by Richael and Gilchrist (15). In the context of systemic movement of the protein, PR1 was discovered first as 15 Kda protein found in plant sap expressed from genetically resistant plants and the gene later cloned by reverse genetics (14). PR1 has leader sequence targeted extracellularly and appears to be quite resistant to proteolytic cleavage due to the fact that it is recoverable in expressed plant sap (14). As a general principle secreted proteins are stable against proteolysis. Given that we have demonstrated that PR1, constitutively expressed in grape confers protection against PD, the question of whether it can protect across a graft union

is a next logical step in protection of cultivated grapes without altering the scion. To this end we have fused the PR1 leader to GFP and confirmed by confocal microscopy that this leader functions to secrete the GFP protein to the apoplast. Also, it should be noted in reference to the the focus on PGIP in the RFP, with emphasis on systemic movement of PGIP across a graft union, that the VvPR1 gene codes for a protein that is approximately one-half the molecular size of PGIP and just as likely to be stable in the apoplast (14). Suggestive of a prophylactic mechanism, using RT-PCR we determine that there is a very slight induction of PR1 mRNA in the susceptible infected plants compared to 1,000-10,000-fold higher expression level in the protected transgenic VvPR1 plants before the plants were inoculated with *Xf.*

cDNA CB456

cDNA 456 from grape is a 350 bp sequence that does not contain a protein coding sequence but does contain a stem/loop structure derived from the 3'UTR of a gene designated as p23 in potato (Figure 2). The p23 gene is reported to be up-regulated in *Meloidgyne incognita*-induced giant cells in tomato and has a protective effect against apoptosis in yeast. The stem/loop structure is quite interesting in the context of suppressing PCD. When we investigated the secondary structure of the sequence by folding analysis with the program mFOLD, the data revealed a striking conservation of the both the stem and loop sequence between cDNA 456 and 3'UTR of Bcl2, a widely studied apoptosis blocking animal gene. Regulation of translation is a reported function of stem/loops found in the 3' UTRs as illustrated by the report by Dr. Martin Dickman's laboratory(18), whereby protection against PCD in plants was due to sequences within 3'UTR of Bcl2. Given that cDNA 456 protection is afforded by RNA (presence confirmed by Northern analysis) and not a translated protein (lack of open reading frame based on comparison to p23) suggests that the PD suppressive action is resident in the 32 bases comprising the cDNA 456 stem and loop sequence that has structural identity to the Bcl 2 human gene's 3'UTR (Figure 2). Our objective is to take the CB456 cDNA-transformed Freedom rootstock, which is protected against PD symptoms, and graft it to untransformed scions of susceptible Thompson Seedless to determine whether susceptible scions can be protected across the graft union. With respect to movement of regulatory small RNAs in plants, Neelima Sinha at UC Davis (13) , has shown plant RNA moves from root to scion in grafted tomato via the phloem to bring about a change in leaf morphology in which the mobile RNA, detected by PCR, induced activation of a transcription factor necessary for the phenotype change.

Publications, reports, and presentations

1. Harvey, J. JW, J. E. Lincoln, K. Zumstein and D. G. Gilchrist 2008 Programmed cell death suppression in transformed plant tissue by cDNAs identified from an *Agrobacterium rhizogenes*-based functional screen. Molecular Genetics and Genomics (in press)
2. Gilchrist, D and J Lincoln. 2007. Evaluation of Genes Isolated by a Functional Genetic Screen for Suppression of Bacterial Growth or Symptoms in Pierce's Disease. Symposium Proceedings, p 18-21, 2007 Pierce's Disease Research Symposium. San Diego, CA December 5-7

Research relevance statement, describing how this research will contribute towards solving the PD/GWSS problem in California

Current literature and results from our laboratory and others indicate a number of plant diseases result from induction of PCD in the host cells in advance of microbial growth (2,15,16). The induction of PCD results in an orderly dismantling of cells that includes maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen, and hydrolytic enzymes have been rendered harmless to contiguous cells. However, when the cell contents are released they can serve as nutrients for microbial cells when they are present in the immediate environment (2,12). Hence, bacteria like *Xf* could receive nutrients from cells adjacent to the xylem when triggered to undergo PCD resulting in gradual release of contents of the grape cell into the apoplastic space surrounding the xylem. The fact that we measure bacterial titers 3-4 orders of magnitude higher in symptomatic grape plants than in either asymptomatic wild grapes or our transgenic asymptomatic grape plants is consistent with enhanced nutrition in the xylem of infected symptomatic plants. The working scenario therefore is; block death, limit death dependent nutrient release and restrict bacteria multiplication. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that are capable of suppression of PD symptoms when constitutively expressed as transgenes. Based on results obtained since September 2007, transgene suppression of PCD affords protection against symptoms and death of grape plants infected with Xf and limits the bacterial titer up to 4 orders of magnitude below that reached in untransformed plants that are killed during infection.

Summary in lay terms of the specific accomplishments of the research project

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program and the NAS report. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will prevent infection, spread or symptom development associated with the presence of *Xf* in the xylem. Recent published information from our laboratory established that susceptibility of several plants to a range of pathogens depends on the ability of the pathogen to directly or indirectly trigger the activation of preformed, genetically determined pathways leading to apoptosis or programmed cell death (PCD) (1,2,3,4,5,6). Using genetic or chemical approaches to block PCD is an emerging and widely reported approach in both animal and plant disease prevention (2,7,8). We have demonstrated that transgenic suppression of PCD via altered expression of endogenous grape genes VvPR1, CB390 (metallothionein) and a regulatory 3'UTR sequence, named cDNA 456 provided protection against symptoms and death of grape plants infected with *Xf*. Furthermore these transgenes limit the bacterial titer up to 4 orders of magnitude below that reached in untransformed plants that are killed during infection.

Summary and status of intellectual property produced during this research project.

Patent disclosure will be submitted within the next month. Awaiting analysis of data related to qPCR of bacterial populations in transgenic Thompson seedless inoculated plants.

Table 1.			
Construct	**Gene**	**Source**	**# independent transformants**
CB390	metallothionein	Grape cDNA library	22
CB456	350bp sequence from 3'UTR of "nematode-inducible" p23 gene	Grape cDNA library	27
CBWG23	cupin-like	Grape cDNA library	23
CBWG71	cytokine-like gene (MIF)	Grape cDNA library	20
CBWG75	germin-like gene	Grape cDNA library	24
CBPR1A	VvPR1	Grape cDNA library	27
CBI35	Intron p35 (anti-PCD control gene)	baculovirus	14
CBP14LD	P14 (homolog of PR1)	tomato	24
CB376	Mycorrhizal induced gene	tomato	27

Table 1. Plant anti PCD genes, from functional screens of cDNA libraries, were transformed into Thompson seedless, 10 clone ramets are being made and inoculations started.

Table 2		
Genotype	**# of Independent Transformants**	**# of Plants**
FR - CBP14	16 (5 tested to date)	293
FR - CB456	7 (2 tested to date)	112
FR – CBGFP control	10 (5 tested to date)	140
FR - CB390	9 (3 tested to date)	126
FR-untransformed control	10 tested this experiment	39
total	**33**	**850**

Table 2. The anti-PCD genes obtained from the cDNA library screen were transformed into cv Freedom and clonally propagated into ramets prior to inoculation. Each of the transgenic plants to be inoculated was trained to two canes and grown to a height of approximately 90 cm prior to inoculation with 40 µl 10^8 cells/ml of GFP-tagged X. *fastidiosa* by stem puncture**.**

Table 3 **Plant name**	**Relevant genotype**	**Disease Category 4-5 plants based on 5 point scale at 6 mo post inoculation**	**Range of bacterial load per gm of stem in asymptomatic category 5 branch at 6 months post inoculation**
CBP14-14	CaMV 35S-driven PR1	90%	10^3
CBP14-13	CaMV 35S-driven PR1	80%	10^4 - 10^5
CBP14-11	CaMV 35S-driven PR1	75%	10^4
CB456-3	CaMV 35S-driven p23, 3' UTR	90%	10^4 - 10^5
CB456-6	CaMV 35S-driven p23, 3' UTR	85%	10^4 - 10^5
CB390-8	CaMV 35S-driven metallothionein	75%	10^4- 10^5
CBGFP	CaMV 35S-driven GFP- transformed and untransformed plants as controls	All dead at 2 months	~10^8 at the time the plants began to die at 2 months post inoculation
V. californica	Asymptomatic wild type untransformed grape host	no death after 12 months post inoculation	10^4

Table 3. Analysis of the disease level in transgenic and non-transformed control plants, based on a 5 point visual rating scale (figure 5) and an assessment by quantitative PCR of the level of bacteria in the vascular system of the inoculated plants in each category and the times indicated. Addition cuttings of these plants are being re-tested under greenhouse inoculation conditions.

Figure 1 Representative Thompson Seedless plants carrying potential anti-PCD genes including cDNA 456, VvPR1 and all other genes listed in Table 1. Approximately 400 plants are currently in the inoculation experiment with *X. fastidiosa*.

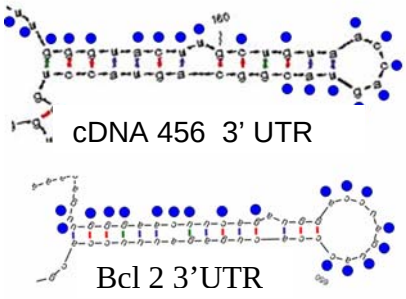
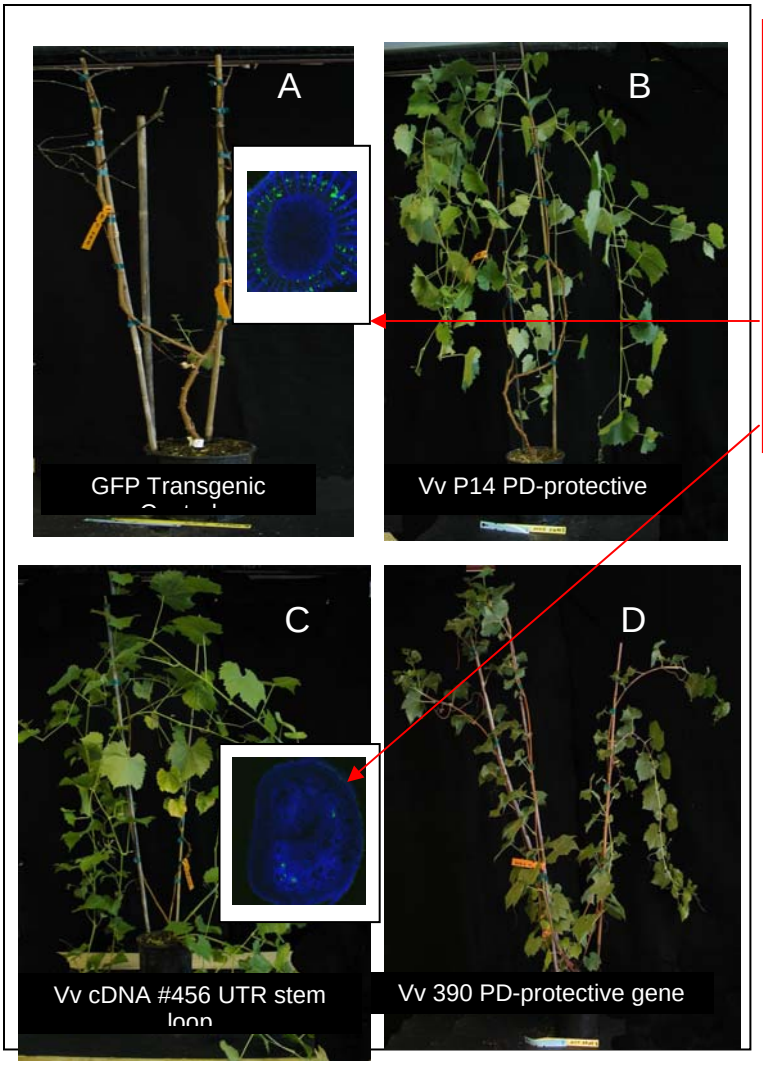


Figure 2 Illustration of the nucleotide conservation between cDNA 456 3'UTR and Bcl 2 3' UTR



Confocal microscope images of inoculated stem cross sections, seen as inserts in panels A and C, illustrate the relative levels of *X. fastidiosa*-GFP in the vascular system of the respective grape lines. These images can be compared to the qPCR quantitative assessment of bacteria in equivalent stem sections presented in Table 3.

Figure 3 Anti-PCD genes expressed in transgenic grape (cv. Freedom) plants suppress symptom appearance in PD susceptible plants without affecting the presence of *X. fastidiosa* in the asymptomatic branches. The transgenic control plants and non-transgenic plants were uniformly dead at 2 months under the same conditions with the same level of initial inoculum. The transgenic asymptomatic resistant plants bearing the respective genes as indicated were photographed at 6 mo.

References:

1. Gilchrist, D. G. 1997. Mycotoxins reveal connections between plants and animals in PCD and ceramide signaling. Cell Death and Differentiation 4:1312-1317.
2. Gilchrist, D. G. 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. Ann. Rev. Phytopathology 36:393-414
3. Richael, C., Lincoln, J., Bostock, R., and Gilchrist, D. G. 2001.Caspase inhibitors reduce symptom development in compatible plant-pathogen interactions and limit pathogen multiplication *in planta.* Physiol. and Mol. Plant Pathol. 59(4) 213-221.
4. Lincoln J.E., Richael, C., Overduin, B., Smith, K., Hall, B.D. Bostock, R.M., and Gilchrist D. G. 2002. Expression of the anti-apoptotic baculovirus p35 gene in tomato results in inhibition of cell death and a decreased susceptibility to a variety of pathogens. Proc Natl Acad Sci. 99: 15217-15221.
5. Harvey, J. JW, J. E. Lincoln, K. Zumstein and D. G. Gilchrist 2008. Programmed cell death suppression in transformed plant tissue by cDNAs identified from an *Agrobacterium rhizogenes*-based functional screen. Molecular Genetics and Genomics (in press)
6. Gilchrist, D., J.E. Lincoln, and C. Richael, 2001. *Inhibiting apoptosis in plants using a baculovirus p35 protease inhibitor gene.* **Patent # 6310273,** United States Patent Office.
7. Greenberg, J.T. and Yao, N. 2004. The role and regulation of programmed cell death in plant-pathogen interactions. Cellular Microbiology. 6:201-211.
8. Nicholoson, D.W. 2000. From bench to clinic with apoptosis-based therapeutic agents. Nature. 407:810-816.
9. Cook, Doug. 2005. Functional genomics of the grape-Xylella interaction: towards the identification of host resistance determinants. 2005 Pierce's Disease Research Symposium. San Diego, CA December 5-7.
10. Gilchrist, D and J Lincoln. 2007. Evaluation of Genes Isolated by a Functional Genetic Screen for Suppression of Bacterial Growth or Symptoms in Pierce's Disease. Symposium Proceedings, p 18-21, 2007 Pierce's Disease Research Symposium. San Diego, CA December 5-7.
11. Lindow, S.E., Newman, K.L., Purcell, A., and Almeida, R. 2002. Management of Pierce's Disease of grape by interfering with cell-cell communication in Xylella fastidiosa. Symposium Proceedings, p 20-22, Pierce's Disease Research Symposium. San Diego, CA December 15-18.
12. Li, C, S J. Barker, D G. Gilchrist, JE. Lincoln and W A. Cowling. 2008. *Leptosphaeria maculans* elicites apoptosis co-incident with lesion formation and hyphal advance in *Brassica napus.* Molecular Plant Microbe Interactions. (accepted)
13. Kim, M, W. Canio, S. Kessler, N. Sinha. 2001. Developmental changes due to long distance movement of a homeobox fusion transcript in tomato. Science, 293; pages 287-289
14. Achim E. Gau1, Mostafa Koutb1, Markus Piotrowski2 and Klaus Kloppstech1 2004. Accumulation of pathogenesis-related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression ofPR genes in the resistant cultivar Remo. *European Journal of Plant Pathology* 110: 703–711.
15. Richael, C. and Gilchrist, D.G. 1999. The hypersensitive response: A case of hold or fold? Physiol. Mol. Plant Pathol. 55:5-12.
16. Wolpert, T. J., L. D. Dunkle, L. Chafetti (2002). "Host-selective toxins and avirulence determinants: what's in a name?" Annu Rev Phytopathol 40: 251-85.
17. Dickman, M. B., Y. K. Park, et al. (2001). "Abrogation of disease development in plants expressing animal anti-apoptotic genes." Proc Natl Acad Sci U S A 98(12): 6957-62.
18. Khanna, H. et al. 2007. Inhibition of Agrobacterium-induced cell death by anti-apoptotic gene expression leads to very high transformation efficiency of banana. MPMI 20:1046-1054