# Looking for LOV: Location of LOV1 function in *Nicotiana benthamiana* cells

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**Abstract:**

To initiate the defense response to an invading pathogen, plants utilize an array of immune receptors to recognize virulence effectors. Virulence effectors are released by pathogens to suppress immune responses in target hosts. These effectors are recognized by a family of resistance proteins known as nucleotide-binding leucine-rich repeat (NB-LRR) proteins. Past research has shown these NB-LRR proteins localize to and exert their function in the cell nucleus through transcriptional re-programming. The *Arabidopsis thaliana LOV1* gene produces a NB-LRR protein that confers sensitivity to the host-selective toxin, victorin, produced by *Cochliobolus victoriae*. When LOV1 is expressed in *Arabidopsis* or *Nicotiana benthamiana*, exposure to victorin elicits the hypersensitive response (HR), which is typically associated with the plant defense response. In this study, we show that LOV1 localizes to and exerts its function in the plasma membrane, and does not require nuclear localization in *Nicotiana benthamiana*. Preventing nuclear entry of LOV1 did not affect its ability to mediate the victorin-induced HR, supporting the hypothesis that LOV1 does not require nuclear localization. Inhibiting de novo transcription of defense genes by use of an adenosine analog, cordycepin, showed no difference in the ability of LOV1 to confer HR cell death, indicating that the LOV1 response does not involve transcriptional reprogramming. Tethering LOV1 irreversibly to the plasma membrane with a dual acylated N-terminal tag did not impact its ability to mediate HR cell death, reinforcing the idea that LOV1 signaling occurs at the plasma membrane. This

research supports the idea that plant NB-LRR proteins can exert their effector-mediated HR function without nuclear localization.

**Introduction:**

Disease in plants caused by pathogens plays a crucial role in the destruction of crops, including the somewhat recent case of the crown rust fungus and Victoria blight on oats (*Avena sativa*). Victoria Blight appeared on oats whose genomes contained the crown rust resistance gene *Pc-2*, which confers resistance to the biotrophic fungus *Puccinia coronata* (Meehan and Murphy, 1947; Wolpert et al., 1985). *Cochliobolus victoriae* is a necrotrophic fungus that incites host cell death during pathogenesis by secreting a toxin, victorin (Meehan and Murphy, 1947; Wolpert et al., 1985). Normally, plants rid themselves of infectious biotrophic pathogens by initiating a form of programmed cell death often called the hypersensitive-response (HR) (Heath, 2000). However, as a necrotroph, *C. victoriae* is able to grow on dead cells, leading certain genotypes of oats and other plants to become susceptible to the fungus through resistance-associated, HR-induced cell death (Wolpert et al., 1985, Wolpert et al., 2002). Susceptibility to Victoria Blight is conditioned in the host cell by a single dominant gene and a single pathogen-derived host-selective toxin (HST) (Lorang et al., 2007). Resistance to a biotrophic pathogen, such as *Puccinia coronata*, on the other hand, is conditioned by a dominant host resistance gene, and dominant avirulence (AVR) gene of the pathogen (Flor, 1942). Resistance gene products are predicted to guard the targets of pathogen

virulence effectors, thereby recognizing the presence of the effector. Recognized effectors thus become AVR determinants. The toxin, victorin, mimics a recognized virulence factor (Van Der Biezen and Jones, 1998). Evidence indicates that Pc-2, which encodes resistance to the rust fungus, and *Vb*, which encodes susceptibility to Victoria blight, are the same gene, implying a relationship between the traits of resistance and susceptibility in plants, and that *C. victoriae* exploits the defense response (Wolpert et al., 2002).

A functional homolog of *Vb*, named *LOV1,* was identified in *Arabidopsis thaliana* (Lorang et al., 2007). *LOV1* encodes a nucleotide-binding-leucine-rich-repeat (NB-LRR) protein, a family of proteins typically associated with disease resistance (Belkhadir et al., 2004; Gao et al., 2011; Lorang et al., 2007). LOV1 is thought to act as a typical disease resistance protein by “guarding” a defense-associated thioredoxin, TRX-h5 (Lorang et al., 2012; Sweat and Wolpert 2007; Wolpert et al., 2002). In *Arabidopsis*, defense against biotrophs is compromised when victorin bind to and inhibits TRX-h5 without LOV1 present. However, this does not result in disease from *C. victoriae* (Lorang et al., 2012). If LOV1 is present, the binding of victorin to TRX-h5 activates LOV1 and elicits a resistance-like response that would confer resistance to biotrophs but confers disease susceptibility to the necrotoph, *C. victoriae* (Lorang et al., 2007).

The purpose of this study was to clarify the mechanism by which the LOV1-TRX-h5-victorin interaction initiates a cell death response. Research has shown that some NB-LRR proteins can function solely in the plasma membrane (Gao et al., 2011), where others are transported to the nucleus to exert their

function (Bai et al., 2012). Specifically, we show that LOV1 localizes to and functions solely in the plasma membrane. We determined the cellular location of the LOV1 protein during activation and signaling of the HR by construction of various tagged LOV1 proteins, imaging with fluorescent confocal microscopy, and by quantitatively monitoring cell death over time by measuring electrolyte leakage. Identifying where LOV1 was located throughout the duration of the victorin-induced response interaction helped to determine where it exerts its function. Understanding where LOV1 functions will help us understand the nature of the cell death regulated by LOV1, and how it confers susceptibility to *C. victoriae*. The experiments were carried out by transiently expressing LOV1 and TRX-h5 proteins in *Nicotiana benthamaiana* leaves.

**Materials and Methods:**

***Plant Growth Conditions****.*

*N. benthamiana* plants used for *Agrobacterium tumefaciens* infilitration, electrolyte leakage assay and confocal microscopy experiments were grown at 24 °C under a 16-h light/8-h dark cycle for three weeks.

***Bacterial Colony Preparation****.*

LOV1 and TRX-h5 genes (Lorang et al., 2007; Sweat and Wolpert, 2007) were cloned into plant transformation vectors (Invitrogen, Carlsbad, CA), and electroporated into *Agrobacterium tumefaciens* GV2260. Transformed *A. tumefaciens* colonies were streaked in plates containing Luria Broth (LB) medium and the antibiotic, kanamycin (Kn). The bacteria were incubated for 48 hours at

37°C. Isolated colonies of both LOV1 and TRX-h5 were placed separately into 15 ml tubes containing 3 ml of LB solution with Kn, and set in a shaker at 37°C for 24 hours. The same procedure was repeated with each genetic construct of LOV1.

***N. benthamiana* Leaf Infiltration**.

The LOV1 and TRX-h5 colonies were grown with shaking for 24 hours at 37°C, removed, at an optical density ~1.2-1.4 (appearing light and foggy), and then transferred into separate 2 ml tubes. These 2 ml tubes were placed into a centrifuge to spin at medium speed for two minutes. After removal from centrifuge, the supernatant was pipetted off to leave only the bacterial pellet at the bottom. The pellet was resuspended in 2 ml of infiltration solution (10 mM $MgCl_2$, 10 mM MES pH 5.3) with a vortex machine. Once resuspended, solutions from each tube were poured into a 15 ml Falcon tube and adjusted to 0.5 O.D with infiltration solution. Acetosyringone was added to a final concentration of 2 μM, and tubes were left to rest on their sides for 2 hours at room temperature to induce *A. tumefaciens* virulence.

The youngest fully-expanded leaf of 3 week old *N. benthamiana* plants was identified for each plant and marked with a black marker. Using a 1 ml syringe with no needle, the bacterial solution was injected into the underside of each marked leaf, so that the solution spread throughout the leaf. After infiltration all plants were placed back in growth room at 24°C for 48 hours.

**Vector Constructs**

Nuclear Exclusion Signal (NES), mutant NES (nes), Calcineurin B–like protein (CBL), and mutant CBL (mCBL) sequences were all engineered as a fusion with the LOV1 protein to study their effect on LOV1-mediated cell death from victorin. These tags (Table 1) were engineered into primers for amplifying the LOV1 gene and used to perform the Polymerase Chain Reaction (PCR). DNA products were purified using gel electrophoresis and inserted into plasmid pENTR™ (Invitrogen) (Lorang et al., 2007). pENTR™ constructs were linearized and cloned into pEarlygate 101 vector using the clonase reaction (Invitrogen). Constructs were then electroporated into *Agrobacterium tumefaciens* GV2260, and grown on LB plates with Kn (50 µg/mL) for future use.

**Table 1:** Vectors used to genetically engineer LOV1 protein

| | |
|---|---|
| NES | 5'-atggacgagctgtacaagaacgagcttgctcttaagttggctggacttgatattaacaag-3' |
| nes | 5'-atggacgagctgtacaagaacgagcttgctcttaaggcagctggagcagatgctaacaag-3’ |
| CBL | 5’-atgggctgcttccactcaaaggcagcaaaagaattt-3’ |
| mCBL | 5’-atggccagcttccactcaaaggcagcaaaagaattt-3’ |

**Electrolyte Leakage Assay**

Small discs (Cork borer #1, 4 mm diameter) of *N. benthamiana* leaves transiently expressing LOV1 or its various constructs and the TRX-h5 protein were collected (n=6) and floated in assay wells filled with 2.5 ml of water with or without victorin (Wolpert et al., 1985). Ion leakage from leaves was measured at indicated time points by taking readings with a conductivity meter measured in microsiemens (µS) (VWR Scientific Model 604).

**Confocal Microscopy**

Confocal fluorescence microscopy was performed on *N. benthamiana* leaves transiently expressing proteins tagged with yellow fluorescent protein (YFP). Images were taken 2-4 hours after victorin infiltration using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany) with an Argon laser and 488 nm excitation. The fluorescent signal from YFP was collected using the beam splitter and filter configuration, HFT 488/543, NFT 490 and BP 505-530. Chloroplast autoflourescence was collected using the same configuration except a LP650 instead of BP 505-530 filter was used. The objective was a Plan-Neofluar 25x/0.8 (Lorang et al., 2012).

**Results:**

**Amount of cell death induced by victorin directly correlated with amount of electrolyte leakage**

The electrolyte leakage assay was run to quantify cell death following exposure to varying concentrations of victorin. As victorin concentration increased, the amount of ions released from the *N. benthamiana* leaf cells expressing LOV1 + TRX-h5 increased (Figure 1). These results were used to choose a concentration of victorin that induced a response that was linear throughout, and induced cell death by 10 hrs. This concentration of victorin (384 ng/ml) was used to induce cell death and monitor LOV1 and its constructs in *N. benthamiana* cells.

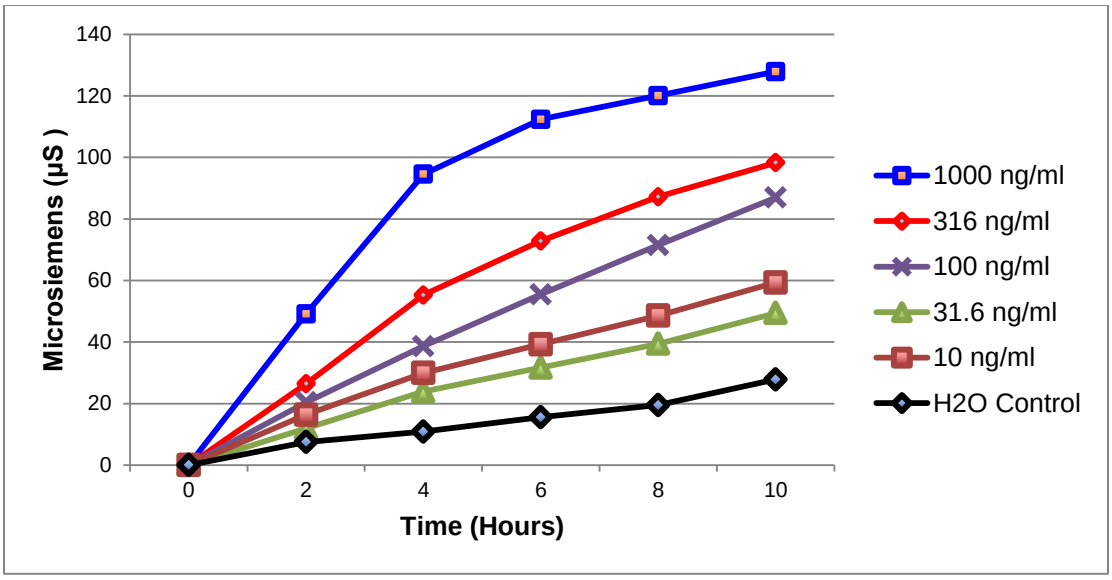


**Figure 1:** Victorin mediated cell death in *N. benthamiana* transiently expressing LOV1 and TRX-h5.

## Exclusion of LOV1 from the nucleus does not block its function

By fusing a nuclear export signal (NES) to LOV1, we demonstrated that LOV1 does not require nuclear localization. To show that the NES tag does not disrupt the function of LOV1, a mutant *nes* tag was used as a control and levels of victorin-induced cell ion leakage were compared between NES-LOV1 and nes-LOV1. *N. benthamiana* leaves expressing NES-LOV1 or nes-LOV1 were treated with water or 384 ng/ml victorin. Three electrolyte leakage assays (ELAs) comparing NES-LOV1 and nes-LOV1 were performed and no significant differences were observed in electrolyte leakage over a 10 hour time course.

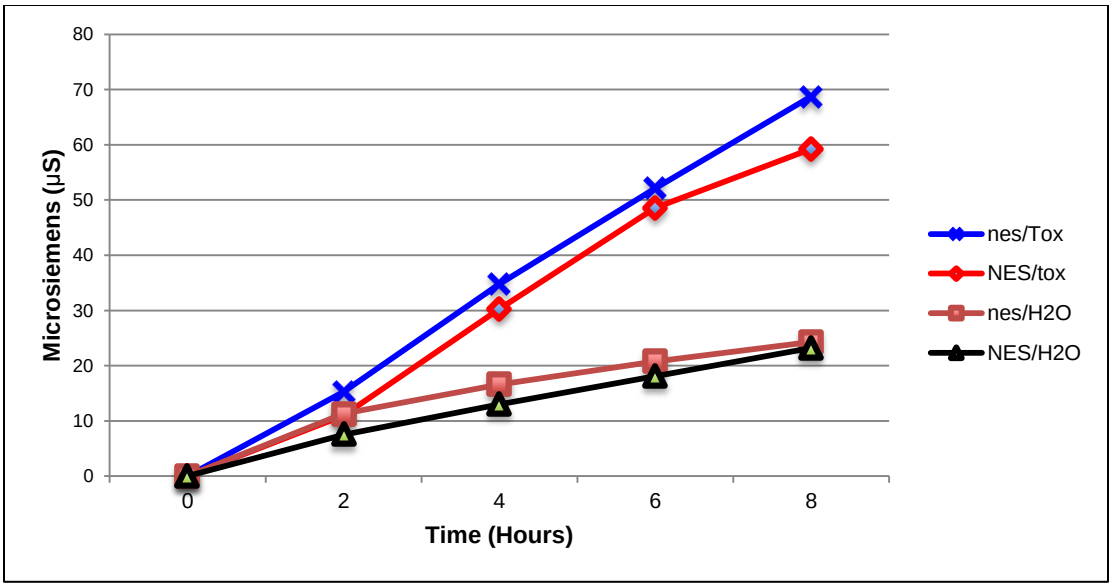


**Figure 2:** Electrolyte leakage assay of *N. benthamiana* transiently expressing TRX-h5 and LOV1 with nuclear export signal (NES) or mutant NES (nes).

## Inhibition of transcription had no impact on electrolyte leakage

To further investigate the requirement for transcription of defense genes during victorin-induced HR, the transcriptional inhibitor cordycepin was included during ELA to inhibit transcription of defense genes. *A. tumefaciens* expressing wildtype *LOV1* and *TRX-h5* genes were infiltrated into *N. benthamiana*, and electrolyte leakage in the presence of 50 µg/mL cordycepin or $H_2O$ and 384 µg/mL victorin or $H_2O$ was measured over time. Figure 3 shows no significant difference in electrolyte leakage between leaves exposed to cordycepin and those exposed to victorin alone.

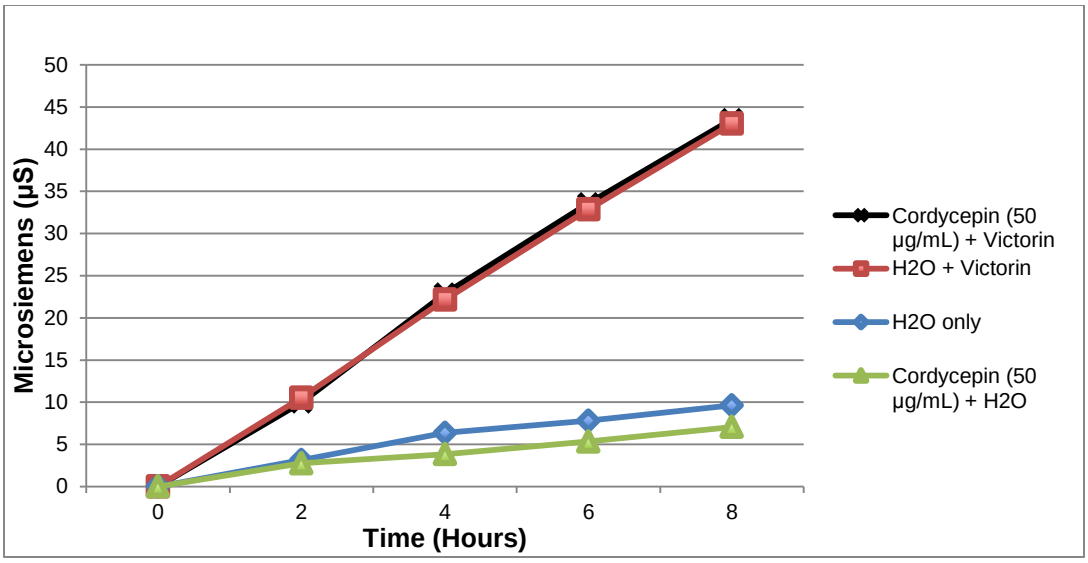


**Figure 3:** Electrolyte leakage assay of victorin-treated (384 ng/ml) *N. benthamiana* transiently expressing WT-LOV1 and TRX-h5 with and without treatment with the transcriptional inhibitor Cordycepin.

## Irreversible attachment to plasma membrane through myristoylation does not affect LOV1 function

A Calcineurin B–like protein (CBL) tag was fused to the N-terminus of LOV1 to irreversibly tether LOV1 to plasma membrane. This is a tag that uses a 12 amino acid peptide to irreversibly tether LOV1 to the plasma membrane through myristoylation, by dual lipid modification (Batistič et al., 2008; Flor, 1942). A mutant CBL-LOV1 (mCBL-LOV1) construct was created to test if LOV1 would still confer sensitivity to victorin with a mutated tag. Using direct symptom analysis, 10 µg/ml victorin was injected into *N. benthamiana* plants transiently expressing TRX-h5 with either CBL-LOV1, mCBL-LOV1, or WT-LOV1 proteins. *N. benthamiana* expressing CBL-LOV1 showed a phenotypic response 30 hours after victorin treatment. Wildtype (WT) LOV1 was used as a positive control. CBL-LOV1 retained HR function, whereas mCBL-LOV1 was not sensitive to

victorin (Figure 4). Results indicated that LOV1 tethered irreversibly to the plasma membrane was still capable of inducing cell death.

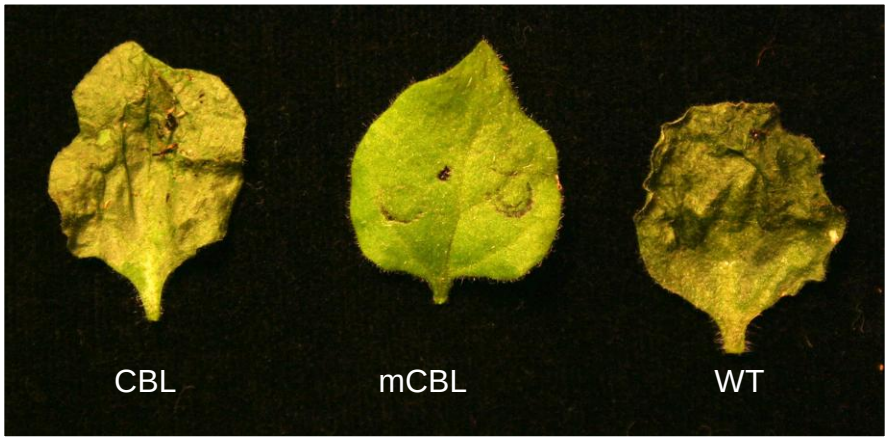


**Figure 4:** Phenotype response of *N. benthamiana* leaves expressing TRX-h5 and LOV1 tethered to the plasma membrane**.** mCBL-LOV1 as control. Photo taken 30 hours post-infilitration with 10 µg/ml victorin.

**LOV1 localizes to plasma membrane of *N. benthamiana* epidermal cells**

Figure 5 shows confocal microscopic imaging of genetic constructs of LOV1/TRX-h5-YFP. Both WT-LOV1-YFP and CBL-cysmut-LOV1-YFP localized at the plasma membrane during activation of victorin-induced HR and throughout a cell-death time course. A cysteine mutation in LOV1 disrupted its localization to the plasma membrane. To test if the CBL tag would restore the localization of cysmut-LOV1, the genetic construct CBL-cysmut-LOV1 was created. The cysmut-LOV1 construct served as a negative control of CBL-cysmut-LOV1.

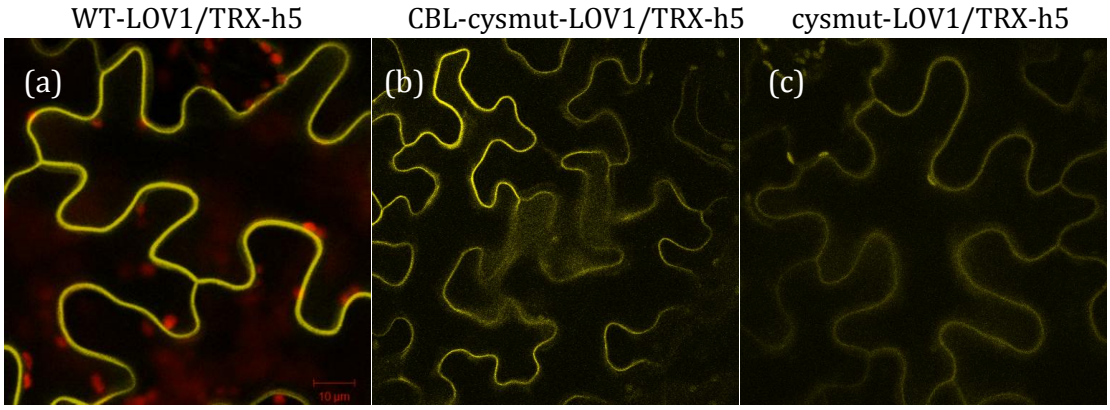


**Figure 5**: (a) Confocal microscopy of yellow fluorescence protein (YFP) tagged WT-LOV1 shown localizing to the plasma membrane of *N. benthamiana* cells. (b) YFP-CBL-cysmut-LOV1 also observed in the plasma membrane. (c) Cysmut-LOV1 used as control for CBL-cysmut-LOV1 construct.

## Discussion:

The initial step in creating a repeatable system for studying the cellular location of functioning LOV1 was to determine an appropriate victorin concentration to use in time course experiments. If too high of a concentration was used, the cells of *N. benthamiana* transiently expressing LOV1 and TRX-h5 would be destroyed before cellular location could be observed. Because 316 ng/ml victorin produced a linear electrolyte response that caused cell death by 10 hours, a concentration of 384 ng/ml was used for future electrolyte leakage assays (Figure 1).

Using a nuclear export signal (NES) tag fused to the C-terminal of LOV1, we were able to prevent LOV1 from entering the cell nucleus. By restricting access to the nucleus, we could test whether nuclear localization is necessary for the victorin-induced HR function of LOV1. A mutant nes tag fusion to LOV1 acted as a control for the functional NES-LOV1 construct. No significant difference was observed in quantitative electrolyte leakage of NES-LOV1 and nes-LOV1 assays

(Figure 2). These data indicate that LOV1 does not require nuclear localization to exert its victorin-induced HR function in *N. benthamiana* cells.

To further interrogate this hypothesis, a transcriptional inhibitor, cordycepin, was used to pre-treat *N. benthamiana* leaves transiently expressing LOV1 and TRX-h5. Because cordeycepin should inhibit newly-induced transcription of defense genes, this analysis provided an alternative approach to determine if victorin-induced HR requires the de novo production of other defense products after LOV1 activation. There was no significant difference in electrolyte leakage between leaves treated with cordycepin and victorin, and those exposed only to victorin (Figure 3). This suggests that the required products to confer effector-mediated HR are pre-formed and remain inactive until virulence effectors are detected. Because experimental data implied that LOV1 is able to operate without nuclear localization and further transcription of defense genes, we wanted to determine if LOV1 could function solely in the plasma membrane.

To test if LOV1 can exert its function solely in the plasma membrane, a calcineurin B–like protein (CBL) tag was fused to the N-terminal of LOV1. We found that both WT-LOV1 and CBL-LOV1 conditioned victorin-induced necrotic cell death, while plants expressing mCBL-LOV1 did not show any symptoms of sensitivity to victorin (Figure 4). The phenotypic response from CBL-LOV1 took approximately 30 hours to show the severe necrotic death, whereas WT-LOV1 showed these symptoms approximately 2 hours post-victorin infiltration (Figure 4). This delay in phenotypic response is likely to be due to the CBL tag being a

N-terminal fusion to LOV1. We previously observed that N-terminal fusions appear to cause LOV1 to adopt a different folding conformation, possibly making it unable to exert the HR function, possibly because it cannot get into the cell membrane. However, since the CBL tag keeps LOV1 in the membrane, it was able to retain its function, whereas the mCBL-LOV1 construct showed no sensitivity to victorin. These data support our hypothesis that LOV1 exerts its function solely in the plasma membrane and does not re-localize to the nucleus to execute the cell-death response.

By adding a yellow fluorescent protein (YFP) tag onto the C-terminus of LOV1 and the CBL construct, we were able to observe LOV1 localization throughout the activation and duration of victorin-induced cell death. Both WT-LOV1-YFP and CBL-LOV1-YFP localized to the plasma membrane of *N. benthamiana* cells (Figure 5). However, it was not clear whether the quantity of LOV1 in the plasma membrane stayed the same throughout the course of cell death.

Heterogeneity among N. benthamiana cells hampered quantifying the amount of LOV1 in the plasma membrane by western blot analysis. Some cells' plasma membranes expressed low levels of fluorescence, from the side effects of *Agrobacterium* and victorin infiltrations, and there was uneven expression of LOV1 throughout the tissue. Plasma membranes of many cells express high levels of fluorescence, but because of this inherent variability, it was difficult to determine if the quantity of LOV1 changed in the plasma membrane based on expression levels of fluorescence.

Our data support the hypothesis that LOV1 localizes to and functions in the plasma membrane. Clarifying how LOV1 confers sensitivity to victorin and consequently susceptibility to Victoria Blight will help understand the mechanism of how R-proteins induce HR in plants. There are still many unknowns regarding the mechanisms by which resistance proteins confer resistance to some pathogens and susceptibility to others. This study showed that LOV1 does not require nuclear localization or de novo transcription after its activation, but can function solely from the plasma membrane.

References:


1. Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., Xiao, W., Liu, Y., Chai, J., Takken, F.L.W., Schulze-Lefert, P., Shen, Q.H. (2012). “Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance”. *PLoS Pathog*. **8**: e1002752.doi:10.1371/journal.ppat.1002752.
2. Batistič, O., Sorek, N., Schültke, S., Yalovsky, S., Kudla, J. (2008). “Dual fatty acyl modification determines the localization and plasma membrane targeting of CBL/CIPK $Ca^{2+}$ signaling complexes in *Arabidopsis*”. *The Plant Cell*. **20**: 1346-1362.
3. Belkhadir, Y., Subramaniam, R., Dangl, J.L. (2004). “Plant disease resistance protein signaling: NBS-LRR proteins and their partners”. *Current Opinion in Plant Biology*. **7**: 391-399.
4. Flor, H.H. (1942). "Inheritance of pathogenicity in *Melampsora lini*". *Phytopath*. **32**: 653–669.
5. Gao, Z., Chung, E.H., Eitas, T.K., Dangl, J.L. (2011). “Plant intracellular innate immune receptor resistance to *Pseudomonas syringae* pv. *maculicola* 1 (RPM1) is activated at, and functions on, the plasma membrane”. *Proc Natl Acad Sci U S A*. **108**: 7619-7624.
6. Heath, M.C. (2000). “Hypersensitive response-related death”. *Plant Molecular Biology*. **44**: 321-334.

7. Lorang, J.M., Sweat, T.A., Wolpert, T.J. (2007). "Plant disease susceptibility conferred by a “resistance” gene”. *Proc Natl Acad Sci U S A*. **104**: 14861–14866.
8. Lorang, J.M., Sweat, T.A., Bradford, C.S., Gilbert, B., Curtis, M., Tzeng, S.-C., Maier, C.S., Wolpert, T.J. (2012). “Tricking the guard: Exploiting plant defense for disease susceptibility”. *Science*. **338**: 659-662.
9. Meehan, F. and Murphy, H.C. (1947). “Differential phytotoxicity of metabolic by-products of *Helminthosporium victoriae”*. *Science*. **106**, 270–271.
10. Sweat, T.A., Wolpert, T.J. (2007). “Thioredoxin h5 is required for victorin sensitivity mediated by a CC-NBS-LRR gene in *Arabidopsis*”. *Plant Cell*. **19**: 673-687.
11. Van Der Biezen, E.; Jones, J. D. G. (1998). "Plant disease-resistance proteins and the gene-for-gene concept". *Trends in Biochemical Sciences*. **23**: 454–451.
12. Wolpert, T.J., Macko, V., Acklin, W., Juan, B., Seibl, J., Meili, J. and Arigoni, D. (1985). “Structure of victorin C, the major host-selective toxin from *Cochliobolus victoriae*”. *Experienta*. **41**: 1524–1529.
13. Wolpert, Thomas J., Laurence. D. Dunkle and Lynda. M. Ciuffetti. (2002). “Host-selective toxins and avirulence determinants: What’s in a name?”. *Annual Rev. Phytopathol*. **40**: 251-285.