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Minireview

# High-throughput functional screening of plant and pathogen genes *in planta*

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**Abstract** Understanding of molecular mechanisms of pathogenicity of *Agrobacterium* and plant viruses has allowed us to utilize these pathogens as efficient vectors for transgene expression in plants. These vectors are now employed for high-throughput overexpression screening of plant and pathogen genes *in planta*. Virus-induced gene silencing (VIGS) has become one of indispensable tools for functional analysis of genes. VIGS is also suitable for high-throughput screening of plant genes. In this review, we describe: (1) vector-mediated transient expression techniques; (2) high-throughput overexpression screening of pathogen genes, of which products elicit hypersensitive response (HR) on host plants; (3) high-throughput overexpression screening of plant genes that are related to HR-like cell death; and (4) high-throughput VIGS screening of plant genes necessary for HR.

**Key words:** *Agrobacterium*, hypersensitive response (HR), overexpression, viral vector, virus-induced gene silencing (VIGS).

The method to randomly express cDNAs *in vivo* and subsequent screening of resulting cells that show phenotypic changes is a common approach to functionally identify novel genes in prokaryotes, yeast and animal cells (Grimm 2004; Rine 1991). High-throughput knockdown screens of mammalian genes by RNA interference have been carried out to identify genes involved in specific signaling pathways (Paddison et al. 2004; Berns et al. 2004). However, such high-throughput approaches for gene function analysis have not yet been fully exploited in plants. In this article, we wish to advocate the application of these approaches to plant systems in that we depict current projects of functional high-throughput screening of plant and pathogen genes. Prefacing we introduce the methods available for the transient overexpression of transgenes in plants by the use of *Agrobacterium* and viral vectors. Finally, latest examples of high-throughput screening by virus-induced gene silencing (VIGS) are introduced.

## Transient overexpression of transgenes using *Agrobacterium* and viral vectors

Overexpression of transgenes in plants has been commonly conducted by either stable or transient transformation. The major advantage of transient transformation is its speed making it appropriate for high-throughput application. So far, electroporation (Lindsey and Jones 1987) and particle bombardment (Klein et al. 1987), both methods requiring special equipment, have mostly been employed for this purpose and the use of suitable vectors for transient overexpression has widely been explored. Although *Agrobacterium*-mediated transformation of plants (Zambryski 1988) is a standard procedure for a long time, only in 1997 an efficient method to transiently express transgenes by infiltration of plant leaves with a suspension of *Agrobacterium* cells carrying T-DNA harboring the transgene has been developed (Kapila et al. 1997). By the method of agroinfiltration whole intact leaves can be easily transformed, in contrast to protoplasts (electroporation) or single cells (particle bombardment), so that the transformed leaves are amenable for subsequent studies (e.g. application of drugs, elicitors or inoculation of pathogens). In Figure 1, the procedure of agroinfiltration is demonstrated.

Brisson (1984) applied for the first time a viral vector (cauliflower mosaic virus: CaMV) to express the dihydrofolate reductase (DHFR) gene *in planta*. Subsequently, tobacco mosaic virus (TMV, Takamatsu et al. 1987; Kumagai et al. 1993), potato virus X (PVX, Chapman et al. 1992; Baulcombe et al. 1995) and other

Abbreviations: PVX, potato virus X; TMV, tobacco mosaic virus; VIGS, virus-induced gene silencing.
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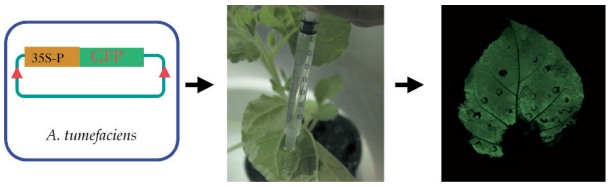


Figure 1. Transient overexpression of foreign genes by agroinfiltration. *Agrobacterium tumefaciens* cells were transformed with a binary vector harboring jelly fish green flurorescent protein (GFP) gene located downstream of a constitutive promoter (CaMV35S-p, left). Liquid culture of transformed *A. tumefaciens* cells were infiltrated to the whole leaf blade of *Nicotiana benthamiana* by a needleless syringe (middle). Two days after infiltration, the leaf blade showed strong green fluorescence under UV lamp, indicating transient production of GFP in the leaf (right).

viral vectors have succesfully been used for rapid transgene expression. Viruses containing transgenes multiplicate in plant cells and are transmitted systemically, so that a high level of gene expression can be attained (Kumagai et al. 1993). Taking advantage of this property, we exploited a PVX system to overproduce antimicrobial defensin of *Wasabia japonica* in *Nicotiana benthamiana*, and characterized its antimicrobial activities (Saitoh et al. 2001). A further progress was the insertion of cDNAs encoding the viral genomes into the Ti-plasmid of *Agrobacterium* so that *Agrobacterium* inoculation could now be employed for efficient virus infection of plants (Turpen et al. 1993). Thus, it is now possible to highly overexpress transgenes by first placing the corresponding DNA sequence in a viral vector located on a Ti-plasmid, followed by infiltration of plant leaves with *Agrobacterium* that harbours this construct. Use of virus-coded suppressors of post-transcriptional gene silencing (PTGS) can further enhance the level of expression (Mallory et al. 2002).

## Screen for pathogen genes causing cell death in plants

Takken et al. (2000) exploited the aforementioned *Agrobacterium*-mediated virus gene expression system to screen *Cladosporium fulvum* cDNAs eliciting hypersensitive response (HR) cell death in tomato plants. For this, messenger RNA was isolated from *C. fulvum*, reverse transcribed to cDNA, and cloned into the binary PVX-based expression vector pSfinx. This cDNA library was transferred to *A. tumefaciens* and 9,600 of the bacterial colonies were individually inoculated by toothpicks onto leaflets of tomato plants resistant to *C. fulvum*.

This screening identified four cDNAs that induced necrotic lesion formation around the inoculated sites. One of the clones encoded the known avirulence factor protein, AVR4, that elicits HR in tomato plants with *Cf4* resistance gene. Qutob et al. (2002) cloned 16 unique cDNAs predicted to code for secreted proteins from *Phytophthora sojae* in the PVX-based binary vector pGR107 and expressed them in *N. benthamiana* leaves, resulting in the identification of the necrosis-inducing factor PsoNIP. Similarly, Torto et al. (2003) cloned 63 cDNAs of *Phytophthora infestans* that coded for putative extracellular proteins into the PVX-based binary vector pGR106. Expression of these proteins in *N. benthamiana* leaves led to the discovery of two novel necrosis-inducing cDNAs, *crn1* and *crn2*. These studies demonstrated that the *Agrobacterium*-mediated virus gene expression system is a powerful tool for high-throughput functional screening of pathogen genes.

## Screening of plant genes causing HR-like cell death in plants

*In planta* high-throughput expression analysis of plant derived cDNAs was applied in an approach to identify cell death-inducing factors. Karrer et al. (1998) constructed a tobacco cDNA library in a TMV-based expression vector and used it to generate infectious transcripts for the inoculation of tobacco plants. By this method 12 different cDNAs that cause HR-like cell death were identified. Their coding sequences showed similarity to ubiquitin, a tumor-related protein and various unknown proteins, respectively. However, in this study, no clear evidence was presented linking the overexpression of particular proteins and the observed cell death. The phenotypes may be caused by co-suppression (ubiquitin gene) or overexpression (tumor-related protein). Recently, we performed a large-scale screening of *N. benthamiana* cDNAs for cell death-causing factors employing the binary-PVX system (Nasir et al. 2005). Messenger RNA was extracted from *N. benthamiana* leaves pretreated with *Phytophthora infestans* elicitor protein INF1 (Kamoun et al. 1998). Double-stranded cDNA was synthesized from the mRNA and subsequently cloned into pSfinx vector (Takken et al. 2000) in the sense orientation, resulting in a cDNA library with more than 100,000 clones. After transforming the library into *A. tumefaciens*, 40,000 colonies were individually inoculated onto leaf blades of *N. benthamiana* by toothpicks. The phenotype around the inoculated site was observed 1–2 weeks following the inoculation (Figure 2). The corresponding *Agrobacterium* clones giving rise to necrotic cell death around the toothpick-inoculated sites were liquid cultured, and infiltrated into leaf blades using needleless syringes to confirm whether the necrotic phenotype was visible over the infiltrated area. By this means it was possible to identify 30 clones which elicited cell death in plant. The nucleotide sequences of the cDNA inserts were determined and BLAST-searched (Altschul et al. 1990) for gene anotation. They included genes coding

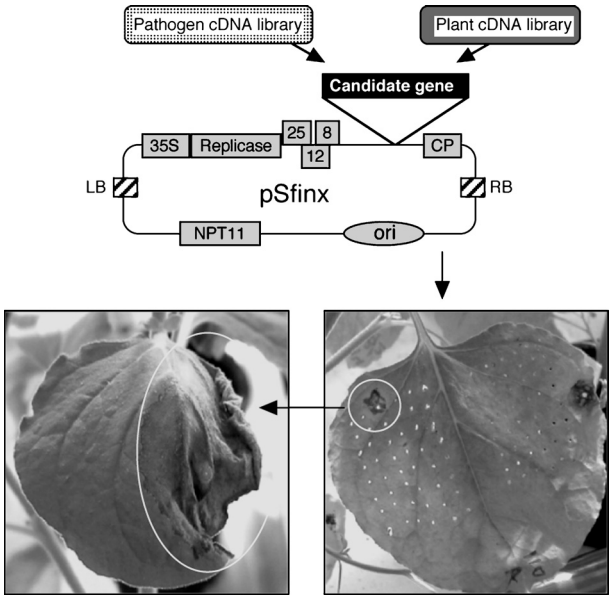


Figure 2. High-throughput screen of HR-like cell-death causing factors in *Nicotiana benthamiana*. A cDNA library was made from plant leaves. Alternatively, pathogen-derived cDNA can be applied. cDNAs were cloned into pSfinx vector (top) and transferred to *Agrobacterium*. *Agrobacterium* clones were toothpick-inoculated onto leaf blades of *N. benthamiana* (bottom right). One to two weeks after the inoculation, necrotic lesions (indicated by a circle) developed around the inoculation sites for a small number of clones. For these clones, *Agrobacterium* were liquid cultured, and infiltrated to right half of the leaf by a needleless syringe to confirm the cell death phenotype (bottom left, indicated by a circle).

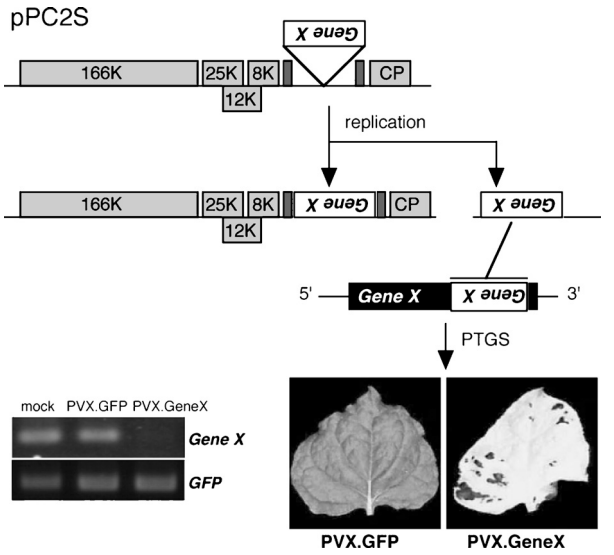


Figure 3. An example of PVX-mediated virus-induced gene silencing (VIGS). A partial cDNA fragment of gene of interest (Gene X) is inserted to PVX vector (pPC2S, Baulcombe et al. 1995). *In vitro* runoff transcript of the vector was inoculated onto *N. benthamiana* leaves to trigger the degradation of endogenous transcripts from Gene X (top). Two to three weeks after inoculation, VIGS of Gene X is established as confirmed by RT-PCR of the Gene X transcript (bottom left) and visible phenotype of the leaves (bottom right). PVX.GFP is used as negative control.

for ubiquitin-like proteins, RNA recognition motif proteins, ethylene-responsive element binding factors (ERFs) and a MAPKK-like protein. We selected an ERF-like protein gene (coined *NbCD1*) for further study as it caused the strongest cell death phenotype. NbCD1 belongs to the class II ERF and has a transcriptional repressor activity via its EAR motif located at the C-terminus. Deletion of the EAR motif abrogated the NbCD1 function as a cell death inducer. Furthermore, *NbCD1* expression was induced by challenge of plants with INF1 elicitor and the non-host pathogen *Pseudomonas cichorii*. PVX-mediated virus induced gene silencing (VIGS see below) of *NbCD1* gene compormised the defense of plants against *P. cichorii*. These results indicate that NbCD1 positively regulates HR-like cell death presumably by down-regulating unknown target genes, and poitively regulates non-host resistance (Nasir et al. 2005). By further extending this screening method to approximately 200,000 clones, we identified more than 100 genes causing cell death on *N. benthamiana* or *N. tabacum* plants after transient overexpression. Functional analysis of these genes is underway.

## Virus-induced gene silencing

As a complement to the screening method by gene-overexpression as described above, screening by gene knock-down employing high-throughput VIGS has been applied to explore genes involved in HR development and plant defense. VIGS is, under normal conditions, specific to viral RNA. However, if the viral RNA is modified to contain a host sequence, the corresponding host RNA is targeted for degradation, thereby causing silencing of the host gene (Kumagai et al. 1995). As VIGS is established in less than one month after inoculation, this is the preferred method to see the loss-of-function phenotype of any gene of interest, and is then suitable for reverse genetics (Baulcombe 1999). In Figure 3, we show the procedure of VIGS using PVX in *N. benthamiana*. Employing the PVX system, we demonstrated that VIGS of *FtsH* gene, which codes for a protease in thylakoid membranes involved in the turnover of photosynthetic protein complexes, causes a striking bleached leaf phenotype in *N. benthamiana* (Saitoh and Terauchi 2002).

Lu et al. (2003) utilized the PVX-mediated VIGS system for high-throughput screening of host genes necessary for HR development downstream of interaction between *Pseudomonas syringae* and its cognate R-gene *Pto*. They cloned a normalized cDNA library of *N. benthamiana* into the PVX vector and individually inoculated 4,992 randomly selected clones

to *N. benthamiana* plants. Three weeks after inoculation, when VIGS of corresponding genes was established, each plant was infiltrated with a mixture of *Agrobacterium* cultures carrying 35S-Pto and 35S-AvrPto constructs, respectively, to trigger the HR. This screening identified 79 clones suppressing the HR. Out of them, three clones coded for a heat shock protein HSP90. VIGS of *HSP90* also compromised HR caused by the interaction of other R-genes and avirulence factors (*Rx*/PVX and *N*/TMV). Thus, Lu et al. (2003) demonstrated that HSP90 is an important component of a R-gene mediated signaling pathway leading to HR. In an almost identical approach del Pozo et al. (2004) identified MAPKKK$\alpha$ as a positive regulator of cell death and resistance against *P. syringae*.

Similarly, Liu et al. (2005) constructed a cDNA library in a tobacco rattle virus (TRV)-based vector and performed VIGS screening of 1,500 cDNAs in *N. benthamiana* plants harboring *N* transgene conferring resistance to TMV. One of the plants exhibited enhanced HR following a challenge with TMV. The cDNA insert of the viral vector inoculated to this particular plant encoded the protein BECLIN1 which is involved in autophagy. Plants deficient in BECLIN1 showed unrestricted run-away cell death upon interaction between host R-gene products and pathogen avirulence factors. The authors suggested that the autophagy machinery comprising BECLIN1 protein acts negatively on the HR programmed cell death in pants.

## Conclusion

Currently high-throughput screening by *in planta* transient overexpression of genes and by VIGS is mainly employed for the identification of plant- and pathogen-derived factors involved in HR and defense. However, with appropriate assay systems for phenotype evaluation, these techniques will easily be adopted for the identification of genes involved in other aspects of plant life. Functional screening of genes as described here recommends itself as an efficient method for hunting novel genes and to further define the cellular pathways they are acting in.

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### References

Altschul SF, Gish W, Miller W, Myers Ewm Kipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410

Baulcombe DC, Chapman S, Santa Cruz S (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J* 7: 1045–1053

Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. *Curr Opin Plant Biol* 2: 109–113

Berns K, Hijmans EM, Mullenders J, Brummelkamp TR, Velds A, Heimerikx M, Kerkhoven RM, Madiredjo M, Nijkamp W, Weigelt B, Agami R, Ge W, Cavet G, Linsley PS, Beijersbergen RL, Bernards R (2004) A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature* 428: 431–437

Brisson N, Paszkowski J, Penswick JR, Gronenborn B, Potrykus I, Hohn T (1984) Expression of a bacterial gene in plants by using a viral vector. *Nature* 310: 510–514

Chapman S, Kavanagh T, Baulcombe D (1992) Potato virus X as a vector for gene expression in plants. *Plant J* 2: 549–557

del Pozo O, Pedley KF, Martin GB (2004) MAPKKK$\alpha$ is a positive regulator of cell death associated with both plant immunity and disease. *EMBO J* 23: 3072–3082.

Grimm S (2004) The art and design of genetic screens: mammalian culture cells. *Nat Rev Genet* 5: 179–189

Kamoun S, van West P, Vleeshouwers VG, de Groot KE, Govers F (1998) Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. *Plant Cell* 10: 1413–1426

Kapila J, De Rycke R, Van Montagu M, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci* 122: 101–108

Karrer EK, Beachy RN, Holt CA (1998) Cloning of tobacco genes that elicit the hypersensitive response. *Plant Mol Biol* 36: 681–690

Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivery of nucleic acids into living cells. *Nature* 327: 70–73

Kumagai MH, Turpen TH, Weinzettl N, Della-Cioppa G, Turpen AM, Donson J, Hilf ME, Grantham GL, Dawson WO, Chow TP, Piatak M, Grill LK (1993) Rapid, high-level expression of biologically active $\alpha$-trichosanthin in transfected plants by an RNA viral vector. *Proc Natl Acad Sci USA* 90: 427–430

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc Natl Acad Sci USA* 92: 1679–1683

Lindsey K, Jones MGK (1987) Transient gene expression in electroporated protoplasts and intact cells of sugar beet. *Plant Mol Biol* 10: 43–52

Liu Y, Schiff M, Czymmek K, Talloczy Z, Levine B, Dinesh-Kumar SP (2005) Autophagy regulates programmed cell death during the plant innate immune response. *Cell* 121: 567–577

Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, Wu AJ, Rathjen JP, Bendahmane A, Day L, Baulcombe DC (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J* 22: 5690–5699

Mallory AC, Parks G, Endres MW, Baulcombe D, Bowman LH, Pruss GJ, Vance VB (2002) The amplicon-plus system for high-level expression of transgenes in plants. *Nat Biotech* 20: 622–625

Nasir KHB, Takahashi Y, Ito A, Saitoh H, Matsumura H, Kanzaki

H, Shimizu T, Ito M, Fujisawa S, Sharma PC, Ohme-Takagi M, Kamoun S, Terauchi R (2005) High-throughput *in planta* expression screening identifies a class II ethylene-responsive element binding factor-like protein that regulates plant cell death and non-host resistance. *Plant J* 43: 491–505

Paddison PJ, Silva JM, Conklin DS, Schlabach M, Li M, Aruleba S, Balija V, O'Shaughnessy A, Gnoj L, Scobie K, Chang K, Westbrook T, Cleary M, Sachidanandam R, McCombie WR, Elledge SJ, Hannon GJ (2004) A resource for large-scale RNA-interference-based screens in mammals. *Nature* 428: 427–431

Qutob D, Kamoun S, Gijzen M (2002) Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J* 32: 361–373

Rine J (1991) Gene overexpression in studies of *Saccharomyces cerevisie*. *Mehods Enzymol* 194: 239–251

Saitoh H, Kiba A, Nishinara M, Yamamura S, Suzuki K, Terauchi R (2001) Production of antimicrobial defensin in *Nicotiana benthamiana* with a potato virus X vector. *MPMI* 14: 111–115

Saitoh H, Terauchi R (2002) Virus-induced silencing of *FtsH* gene in *Nicotiana benthamiana* causes a striking bleached leaf phenotype. *Genes Genet Syst* 77: 335–340

Takamatsu N, Ishikawa M, Meshi T, Okada Y (1987) Expression of bacterial chloramphenicol acetyltransferase gene in tobacco plants mediated by TMV-RNA. *EMBO J* 6: 307–311

Takken FLW, Luderer R, Gabriels SJEJ, Westerink N, Lu R, de Wit PJGM, Joosten MHAJ (2000) A functional cloning strategy, based on a binary PVX-expression vector, to isolate HR-inducing cDNAs of plant pathogens. *Plant J* 24: 275–283

Torto TA, Li S, Styer A, Huitema E, Testa A, Gow NAR, van West P, Kamoun S (2003) EST mining and functional expression assays identify extracellular effector proteins from the plant pathogen *Phytophthora*. *Genome Res* 13: 1675–1685

Turpen TH, Turpen AM, Weinzettl N, Kumagai MH, Dawson WO (1993) Transfection of whole plants from wounds inoculated with *Agrobacterium tumefaciens* containing cDNA of tobacco mosaic virus. *J Virol Methods* 42: 227–239

Zambryski P (1988) Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annu Rev Genet* 22: 1–30