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RESEARCH PAPER

Mechanism Analysis of Broad-spectrum Disease Resistance Induced by Expression of Anti-apoptotic *p35* Gene in Tobacco

Zhihua Wang[1, 2], Jianhua Song[1, 2], Yong Zhang[1, 2], Baoyu Yang[1], Yao Wang[1], and Shiyun Chen[1]

1 State Key Laboratory of Virology, Wuhan Institute of Virology, the Chinese Academy of Sciences, Wuhan 430071, China
2 Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

Abstract: Studies have shown that transgenic plants expressing antiapoptotic genes from baculovirus and animals increase resistance to biotic and abiotic stress. However, the mechanism under these resistances is conjectural, or in some cases, even controversy. In the present study, the *p35* gene from baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) was expressed in tobacco, and for the first time, P35 protein was detected in transgenic plants by Western blotting. Inoculation of T1 transgenic tobacco leaves with tobacco mosaic virus (TMV) showed enhanced resistance, and DNA laddering was observed after TMV infection in control but not in transgenic plants. DAB staining showed that TMV infection did not affect peroxide induction of transgenic plants; Western blotting analysis of PR1 protein also showed no difference of control and transgenic plants. Inoculation of fungus (*Sclerotinia sclerotiorum*) using a detached leaf assay showed enhanced resistance of transgenic leave tissue. RT-PCR analysis demonstrated that *p35* gene expression induced earlier expression of PR1 gene after *S. sclerotiorum* infection. Taken together, our results suggest that the mechanism under enhanced disease resistance by P35 protein is possibly related to the activation of PR-related proteins in addition to the inhibition of programmed cell death, depending on the pathogens challenged.

Keywords: antiapoptotic, baculovirus, P35, pathogenesis-related protein

Introduction

Plant defense mechanisms against invading pathogens often include rapid cell death, known as the hypersensitive response (HR). HR is in association with rapid and localized cell death at the infected sites of host tissues and plays a role in preventing growth and spread of pathogens into healthy tissues[1]. HR is a form of programmed cell death (PCD), the genetically controlled suicide of cells. Apoptosis is a type of PCD with specific biochemical characteristics[2]. The tight regulation of this program is essential to ensure that it is only activated in the required cells at the proper moment. In addition to these local responses, the uninfected portions of the plant usually develop systemic acquired resistance (SAR), which is manifested as enhanced resistance to a subsequent challenge by the initial or even unrelated pathogens, and is often associated with pathogenesis-related (PR) gene expression[3].

In plants, apoptosis occurs morphologically similar to that of animals, and induces characteristic morphological and biochemical alterations in cells[4,5]. Plant proteases that are functionally equivalent to animal caspases are also reported[6–9]. Expression of antiapoptotic genes in plants has demonstrated to induce broad-spectrum resistance to

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Corresponding author: Shiyun Chen. Tel: +86-27-87199354; Fax: +86-27-87199354; E-mail: sychen@wh.iov.cn

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biotic and abiotic stress, e.g. plant pathogens, wounding, salt, cold, UV-B, and herbicide treatments[8–13]. In one study, plants expressing human *Bcl-2* and *Bcl-xl*, nematode *CED-9*, or baculovirus *IAP* genes all conferred heritable resistance to three necrotrophic fungal pathogens and a virus, and DNA laddering occurs in susceptible but not resistant transgenic tobacco tissue during the normal course of infection by a fungal pathogen *Sclerotinia sclerotiorum*, suggesting that disease development requires host-cell death pathways[12]. In another study, expression of *bcl-xL* and *ced-9* in tomato enhances tolerance to both virus-induced biotic stress and cold chilling-induced abiotic stress[9]. Using chloroplast- directed herbicides to treat tobacco plants, wild-type plants died with features associated with apoptosis, while transgenic plants expressing *bcl* genes survived and did not show any apoptotic-like characteristics, suggesting that the chloroplast served as a location for these animal anti-apoptotic proteins in addition to the established mitochondrial location[10]. All these studies suggest that the function of antiapoptotic genes is to inhibit cell death and thus increase resistance to biotic and abiotic stress.

Similar to antiapoptotic genes from animals, the *p35* gene from baculovirus has also been shown to inhibit caspases in host cells, and suppresses apoptosis[14–16]. *In vivo* expression of *p35* in transgenic systems results in resistance to diverse types of induced apoptosis and delay of cell death caused by both developmental and pathological signals in nematodes[17], Drosophila[18], and mammals[19,20]. These studies suggest that *p35* gene-induced apoptotic regulation is conserved across the kingdoms. Early research in insect cells showed that P35 functions directly as an antioxidant by mopping out free radicals and consequently prevents cell death by acting at an upstream step in the reactive oxygen species-mediated cell death pathway[21]. However, the action mechanism of these antiapoptotic genes in plants is conjectural. Expression of *p35* gene in tomato plants has demonstrated to suppress cell death caused by either a fungal toxin or infection of certain bacterial and fungal pathogens[8]. Infection of *p35*-expressing tobacco plants with tobacco mosaic virus (TMV) disrupts N-mediated disease resistance, causing systemic spreading of the virus within a resistant background, while plants expressing mutant variants of the P35 protein do not show inhibition of HR cell death or enhanced virus systemic movement, suggesting that *p35* gene expression in tobacco delays HR induced cell death, which provides evidence for the participation of caspase-like proteases during HR response[22].

In this report, we have shown that the expression of baculovirus *p35* gene in tobacco led to the protection against diseases caused by a plant virus and a fungus. We also provide evidence that the mechanism under disease resistance is dependent on the pathogens challenged.

1 Materials and methods

1.1 Binary vector construction and plant transformation

The coding region of the *p35* gene was cloned by PCR with the wild-type *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) DNA as template. The following primers were used: 5′-ATTAGATCTCGTTGC TTGTATTAAGTGAGAGC-3′ and 5′-GGCGGTCACCT GATCGTGCATTACAAGTAGA-3′.The PCR product was sequenced and subsequently cloned into the pGEMT-easy vector, and the *uidA* gene in the binary vector pCAMBIA1305 (purchased from CAMBIA, Australia) was replaced by the *p35* gene by restriction with *Bgl* II and *Bst*E II. The *p35* gene was then under the control of the cauliflower mosaic virus *35S* subunit and the nopaline synthase terminator. *Agrobacterium tumefaciens* strain EHA105 containing the binary construct was used to transform leaf discs of tobacco (*Nicotiana tabacum* cv. Xanthi) following the standard protocol[23]. Primary transgenic plants were selected on MS medium containing 30 mg/L hygromycin. Hygromycin-resistant plants were planted in the greenhouse and set seeds. T1 transgenic tobacco plants with segregation against hygromycin as well as the empty vector control plants were grown at 25°C with 16 h light periods in a growth chamber and used for subsequent studies.

1.2 Confirmation of transgenic plants

Integration of the *p35* gene into tobacco genome was confirmed by PCR. Briefly, genomic DNA was isolated from leaves of 8-week-old greenhouse-grown T1 tobacco plants, and the presence of the *p35* gene was amplified by PCR using primers used for *p35* gene cloning.

Expression of P35 protein in transgenic tobacco plants was analyzed by Western blotting. T1 tobacco fully expanded leaves were homogenized in liquid nitrogen by grinding with small plastic pestles in extraction buffer[24], and resuspended in cold extraction buffer (25 mM Tris pH 7.0, 50 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM phenyl-methylsulfonyl fluoride, 2 g/mL aprotinin, 2 g/mL pepstatin A, and 2 g/mL leupeptin). The protein concentration was measured according to the method of Bradford using the Bio-Rad reagent with BSA (Sigma) as a standard. For immunoblot analysis, samples were boiled for 10 min in sample buffer, and proteins were separated by 10% SDS-PAGE and subsequently blotted onto PVDF (polyvinylidene difluoride) membrane (Millipore) by semidry electroblotting. After being blocked for 1 h in Tris-buffered saline (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) with 5% nonfat dried milk at room temperature, the membrane was then incubated for 1 h with

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