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# Production and characterization of transgenic Citrus plants carrying *p35* anti-apoptotic gene

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ABSTRACT

Transgenic plants of Citrus rootstock Carrizo citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] expressing a *p35* gene from AcMNPV (*Autographa californica* moth Nuclear polyhedrosis virus) were produced. The *p35* gene encodes a baculovirus protein which is suggested to be a negative regulator of apoptosis. Transgenic Carrizo plants carrying *p35* gene in their genome had general phenotypic characteristics unchanged from wild-type (WT) plants. This includes tree height, shape, size of leaves, thorniness, levels of gas exchange, and levels/balance of major leaf plant hormones. Transgenic plants also retained their ability to respond to ethylene. Regarding the process of leaf senescence, all three transgenic lines were significantly slower as compared to the WT plants. Explants from two out of three transgenic lines sustained the treatment with *Agrobacterium* better than the explants from WT plants and exhibited higher shoot regeneration rate. For all three transgenic lines, transformation success rate was significantly lower than for the WT material. All these data taken together confirm that the viral *p35* gene can exert its function when it is expressed in citrus plants and make them go through death-associated processes at a slower rate.



## 1. Introduction

Anti-apoptotic genes are negative regulators of apoptosis (Steller 1995; Villa et al., 1997) and are known to induce pleiotropic effects when inserted into the genome of transgenic plants. Apoptosis is an example of programmed cell death that includes marginalization of chromatin in the nucleus, disruption of nucleus and cytoplasm into small sealed packages, and processing and fragmentation of the DNA at nucleosome linker sites (Pennell and Lamb, 1997). Introduction of anti-apoptotic genes such as *p35*, *Bcl-xl*, *Bcl-2*, *ced-9*, and *op-iap* into plants resulted in higher tolerance to environmental stress as well as resistance to some pathogens (Dickman et al., 2001; Lincoln et al., 2002; Awada et al., 2003; Xu et al., 2004; Wang et al., 2009). The p35 baculovirus protein has been shown to inhibit caspases, enzymes that belong to the group of cystein proteases with the function in cleavage of several substrates involved in maintenance of cellular homeostasis (Reidl et al., 2001; Lincoln et al., 2002). Inhibition of caspases taking place during viral infection prevents the death of host insect cells before viral replication is complete. When expressed in tomato, *p35* gene blocked cell apoptosis and caused those plants to be resistant to a variety of pathogenic fungi (Lincoln et al., 2002). Transgenic tobacco plants carrying *p35* gene had enhanced tolerance to salt and oxidative stress as well as starvation (Wang et al., 2009). The products of genes *ced*-9, *bcl-2*, and *bcl-xl* are all involved in the cell death pathway as negative regulators of processes recognized as a part of apoptosis (Dickman et al., 2001). Sequence data point to functional conservation of these genes in the animal kingdom as *ced-9* gene from worm *Cenorhabditis elegans* is homologous to *bcl-2* and *bcl-xl* from humans. Constitutive expression of *bcl-xl* (from chicken), and *bcl-2* in transgenic tobacco resulted in improved response to drought stress. Plants transformed with *bcl-2* started losing turgor at significantly lower levels of relative water content than control plants and plants transformed with both *bcl-xl* and *bcl-2* started losing turgor at lower values of osmotic potential (Awada et al., 2003). Tomato plants transformed with *bcl-xl* and *ced-9* genes exhibited higher tolerance when exposed to cold stress and some pathogens (Qiao et al., 2002; Xu et al., 2004). Cell death leading to cold-induced necrosis of leaves was present more prevalently on control than on transgenic plants (Xu et al., 2004). Co-incubation of plant cells from cell cultures with *Agrobacterium tumefaciens* caused a large percentage of cells to die (Hansen 2000; Khana et al., 2007); how-

*Abbreviations:* ABA, abscisic acid; HLB, huanglongbing-"greening" disease; *tr*-Z, *trans*-Zeatin; WT, wild-type.

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ever, those cells that were transformed with the *p35*, *ced*-9, *bcl-xl,* and *bcl-2* 3′ untranslated regions had a high rate of survival.

Within the last decade, introduction of different types of genes into various members of the Citrus group resulted in improvement of tolerance to environmental and biotic stresses (Orbović and Grosser, 2015). Recent outbreaks of Citrus canker and "greening" disease (HLB—huanglongbing), and lingering presence of Citrus Tristeza Virus (CTV) have put immense pressure on the Citrus industry and researchers all around the world are trying to create transgenic lines that would be either tolerant or resistant to these three diseases and other pests that prevent or decrease profitability. Most recently, it was reported that 25–30% of roots of Citrus trees infected with the HLB have already decayed before any visible symptoms of the disease were visible on aerial parts of the trees (Johnson et al., 2013). This report not only directed the attention of the research community towards roots as very important elements of the infection process but also raised the need for rootstocks with improved tolerance to HLB and increased ability to survive challenging periods of starvation due to decreased photosynthetic capacity of foliage. By following this line of thought, production of transgenic Citrus rootstock plants that carry members of the gene family that would either slow down or in some cases even prevent the death of root cells would be extremely beneficial. Such genes are anti-apoptotic genes and production of transgenic Carrizo rootstock plants (already known not to show strong symptoms of HLB infection on aerial organs; Folimonova et al., 2009), as well as other rootstock varieties, should be one of the priorities.

Our first goal in this project was to produce transgenic Carrizo plants carrying *p35*, one of the known anti-apoptotic genes, and examine the effects of expression of such a gene on the phenotype of citrus plants. These transgenic rootstock plants would need to have important commercial traits unaffected by *p35* before they could be used in tests for resistance to pathogens and abiotic stress.

## 2. Materials and methods

### 2.1. Production and growth of transgenic and WT plants

The gene for the baculovirus p35 caspase inhibitor protein containing the PIV intron (in vector pBSI35) was a gift from Dr. David Gilchrist (University of California, Davis). *BglII* and *SpeI* sites were inserted at the 5′ and 3′ ends of *p35* gene by PCR. Enzyme digested *p35* was ligated into *BglII* and *XbaI* sites of digested pBI524 downstream of the double 35S AMV promoter and upstream of the NOS terminator. This cassette was removed by digestion with *EcoRI* and *PstI* and ligated into *EcoRI* and *PstI* sites of pCAMBIA2301 binary vector.

Transgenic plants of Carrizo citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] were produced using methodology described by Orbović and Grosser (2015). Shoots generated on explants not treated with the *Agrobacterium* were considered as WT. Upon transfer to soil, plants were grown in laboratory for about two months before being moved to the greenhouse. Environmental conditions in the greenhouse were as follows: light intensity ~1300 μmol/m$^2$s, temperature 32 °C/21 °C, and relative humidity 50–90%. All transgenic lines as well as WT plants were propagated in multiple copies. For multiplication of mother plants, one or two branches (overall length of 80–90 cm) were removed from plant and cut into explants (3–4 cm long) with two leaves. Lower sides of the explants were dipped into rooting powder [IBA-indo 3-butyric acid (Rootone, GardenTech, Palatine, IL)] and placed into containers with soil. Explant trays were put onto a "mist-bed", a bench with automated micro-sprinkler system that was spraying water for 10 s in intervals of 3 min. After about one month under these high humidity conditions, 95% of explants grew roots and sprouted new shoots. Within an additional 2 months, propagated plants were about 25 cm tall and most of them had multiple branches.

### 2.2. Morphological features of transgenic plants and WT plants

#### 2.2.1. Observations of senescence

Branches with young leaves were plunged into a tube with water within 1–2 s after they were severed from the plant. No additives were supplied to plants throughout the observation period. The care was taken to have enough water in tubes so that the bases of branches were never exposed to air. Branches were observed for 9 weeks and photographs of senescing plants were taken once a week (Fig. 1). No quantitative measurements of senescence were done during this period that served for visual selection of transgenic lines exhibiting phenotypic differences in comparison to WT plants.

#### 2.2.2. Leaf surface area measurements and counting of stomata

The surface area of leaves was measured with the use of ImageJ software package (NIH—National Institute of Health). Populations of leaves were positioned on the white sheet of paper, pressed with the Plexiglas plate to make leaves flat, and photographed. No plug-ins for the program were used. After importing photographed image into the program, the calibration was done so that the measurement can be performed in appropriate units. The scale line on the image was delineated on the screen with the cursor and by using Analyze → Set scale function, the number of pixel presented by the program was assigned the number of centimeters which in our case was one. Following calibration, the image was transformed with the Image → Adjust → Threshold function so that leaves on the image turned red. Such processing was the result of default function offered by the program. The next step was to do surface area measurements. The table to insert the measurements was open through using Analyze → Tools → ROI manager function. By using the "wand" icon and clicking on leaf images, their surface area was added into the table. By clicking on Measure button available on the data table, the measurements were converted from number of pixels into square centimeters. Stomata were counted from scanning electron micrographs taken as described by Orbović et al. (2001). Twenty micrographs of leaf areas from each genotype were used to count the number of stomata.

### 2.3. Physiological features of transgenic plants and WT plants

#### 2.3.1. Gas exchange measurements

Net gas exchange parameters [$CO_2$ assimilation rate ($ACO_2$), leaf transpiration rate ($E_{lf}$), stomatal conductance ($g_s$), and water use efficiency (WUE)] were determined with a LI-COR portable photosynthesis system (LI-6400; LI-COR Inc., Lincoln, NE). All measurements were taken in the morning from 9:00 to 10:30 a.m. to avoid high afternoon temperatures and low humidity, which can reduce net gas exchange (Jifon and Syvertsen, 2003). Photosynthetically active radiation (PAR) was set to 1000 μmol m$^{-2}$ s$^{-1}$, leaf temperature was 30 ± 2 °C, and vapor pressure deficit of 2.4 ± 0.4 kPa within the cuvette. Fully expanded young leaves from the group of plants that were 6–18 months old were used for these measurements.

#### 2.3.2. Hormone analyses

Leaf tissue harvested from WT and transgenic plants (160–170 mg) was analyzed for the presence of *trans*-Zeatin (*tr*-Z) and abscisic acid (ABA). Samples were taken from four different plants for each genotype tested. Analyses were done with the use of Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Equipment that was employed included 4000 QTRAP system (Applied Biosystems, Carlsbad, CA) coupled with the NexeraX2 (Shimadzu Corp., Kyoto, Japan) chromatograph. The