

## ABSTRACT

Citrus represent the main fruit crop in Spain and in the world. Breeding programs are needed in order to obtain high quality competitive varieties. Traditional breeding of citrus is a slow and complicated process due to their complex reproductive biology and long juvenile periods; therefore, new technologies are necessary to speed up this process. The availability of citrus genome sequences and gene-expression patterns may help identifying candidate genes potentially involved in a particular biological process; however, further analyses are required to associate each gene with a specific phenotype or biological function.

Virus induced gene silencing (VIGS) using viral vectors has been shown to be a helpful tool to evaluate plant gene function by reverse genetics. This technology has advantages respect to traditional methods like mutagenesis and genetic transformation, because it allow us to study the function of many genes in a short time. This is important in the case of citrus, whose juvenile period is often more than 6 years and genetic transformation of adult plants is difficult. Besides, VIGS allows studying genes whose function is essential for plant viability, as these are silenced after the plant has already grown.

At the beginning of this thesis, a viral vector based on the *Citrus tristeza virus* (CTV) genome was available to express foreign proteins, but it was not tested for VIGS analysis. In our laboratory, a cDNA infectious clone was developed of the *Citrus leaf blotch virus* (CLBV) genome, a virus that causes asymptomatic infection in most of the citrus varieties tested. This infectious clone has been modified in order to get viral vectors that can be used for expressing foreign proteins or for citrus gene silencing. For this purpose, a *Pml*I restriction site was engineered at two different positions in the CLBV genomic (g)RNA: at the 3' untranslated region (UTR) (vector *clbv3'*) or at the intergenic region between the movement (MP) and the coat (CP) protein genes (vector *clbvIN*). In order to express foreign sequences by formation of a new subgenomic (sg)RNA, the minimum promoter sequence of the CP sgRNA was delimited by cloning different sized fragments in the *clbv3'* vector. A minimum 92-base fragment between positions -42 and +50 around the transcription start site of the CP sgRNA was shown to contain all the elements required for full promoter activity *in vivo* out of its natural context. This minimum sequence was cloned in the *clbv3'* and *clbvIN*

vectors and both were able to produce a new sgRNA and express recombinant proteins.

The efficiency and stability of these vectors was assessed by inserting different sized linear sequences, or inverted repeats that upon transcription fold as an RNA hairpin, and then inoculating *N. benthamiana* and citrus plants with these constructs. Analyses of viral replication and insert integrity showed that all the constructs based on *clbv3'* were stable in successive flushes for at least three years. However, viral replication was not detected for any of the constructs based on *clbvIN*. Stability of the constructs based on vectors with the duplicated promoter depended on the insert size. Viral replication was always detected but recombination events were observed when the cloned fragments were longer than 720 nt for the *clbvINpr* vector or 408 nt for the *clbv3'pr*.

An important factor to determine efficiency and functionality of the developed vectors is to know their movement and spread patterns in the plant tissues. For this purpose, *N. benthamiana* and citrus plants were inoculated with *clbv3'pr*-GFP, a construct expressing the green fluorescent protein (GFP) in the tissues where it replicates. In *N. benthamiana* plants, observation of GFP allowed CLB detection in most of tissues, the virus accumulating preferentially in ovules and meristematic regions. GFP could not be observed in citrus but the virus was detected in meristematic regions by real time RT-PCR and molecular hybridization. These results explain in part the difficulty to eliminate CLB by shoot-tip grafting *in vitro*.

In order to evaluate the ability of *clbv3'pr* and *clbvINpr* to express proteins, the complete ORF of the *gfp* gene was cloned and the amount of synthesized GFP was quantified in the infected plants. In *N. benthamiana* plants, the amount of GFP produced by the *clbv3'pr* vector was 16  $\mu\text{g}\cdot\text{g}^{-1}$  of fresh tissue, about 5 to 6 times more than that produced by the *clbvINpr* vector. In citrus, this latter vector produced only 0.6  $\mu\text{g}$  of GFP per gram of fresh tissue, whereas no GFP was detected with *clbv3'pr*-GFP due to the instability of the construct in this host.

The VIGS silencing effectiveness of the vectors *clbv3'*, *clbv3'pr* and *clbvINpr* was assayed by cloning endogenous gene fragments (*pds*, *actin*, *sulfur*) as well as the *gfp* gene, experimentally introduced in transgenic citrus plants. In citrus, all the constructs based on the three vectors induced a silencing phenotype for the genes studied, with *clbv3'* vector being the most effective for VIGS analyses

in this host. However, in *N. benthamiana*, silencing was induced only in plants inoculated with the *clbv3'pr*-hp58PDS construct, which expresses a hairpin with a 58-nt inverted repeat of the *pds* gene. All the silenced plants showed a reduction of the cognate mRNA in the plant and an accumulation of siRNAs derived from the inserted gene and from the CLBV gRNA. On the other hand, the silencing phenotype of the studied genes was observed in successive flushes, which confirms the great stability of the CLBV-based vectors.

Viral vectors developed in this thesis provide an efficient tool for gene function studies through reverse genetics using the VIGS technique. They can also be used in forward genetic studies through protein expression or in plant protection against virus, bacteria or fungal diseases or invertebrate pests.