

Virus Induced Gene Silencing of prolyl-4-hydroxylase Induce alteration in Plant Growth

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Abstract: Prolyl 4-hydroxylases (P4Hs) belong to the family of 2-oxoglutarate-dependent dioxygenases and catalyze the formation of 4-hydroxyproline, requiring 2-oxoglutarate and O_2 as co-substrates and Fe^{2+} as a co-factor. Nine putative P4Hs have been identified up to now in the tomato genome. A reverse genetics approach, Virus-Induced Gene Silencing, was used to silence two P4Hs, P4H3 and P4H8. Transient silencing of P4H3 and P4H8 altered tomato plant growth. P4H3- and P4H8-VIGS plants had shorter stems, reduced fresh weight and smaller leaves. The effect of VIGS application in different plant organs such as root, shoot, and root fresh weight, shoot fresh weight, leaves area were analyzed. Linear correlation was measured by calculating the Pearson correlation coefficient in order to see if there are correlations between these variables. These results indicate that P4H3 and P4H8 play a significant role in tomato plant growth and VIGS is a useful tool to study the function of tomato gene families such as P4Hs in growth and development, and elucidate, in the long run, the physiological significance of substrate-proteins such as hydroxyproline-rich glycoproteins.

Key word: prolyl-4-hydroxylase, plant growth, correlation, regression

1. INTRODUCTION

Prolyl 4-hydroxylases (P4Hs) catalyze the formation of 4-hydroxyproline and belong to the family of 2-oxoglutarate and non-heme-Fe(II)-dependent dioxygenases (2-ODDs). P4Hs require Fe^{2+} as a cofactor and O_2 and 2-oxoglutarate as cosubstrate for their catalytic activity (Figure. 1.1) [1]. P4Hs have

been found in vertebrate, invertebrate and plant organisms. In mammalian cells, 4-hydroxyproline is abundant in collagen and collagen-like proteins, while in plants there is a superfamily of cell wall glycoproteins, rich in hydroxyproline, collectively called hydroxyproline-rich glycoproteins (HRGPs). P4Hs have been cloned and characterized from

Caenorhabditis elegans [2] and *Drosophila melanogaster* [3].

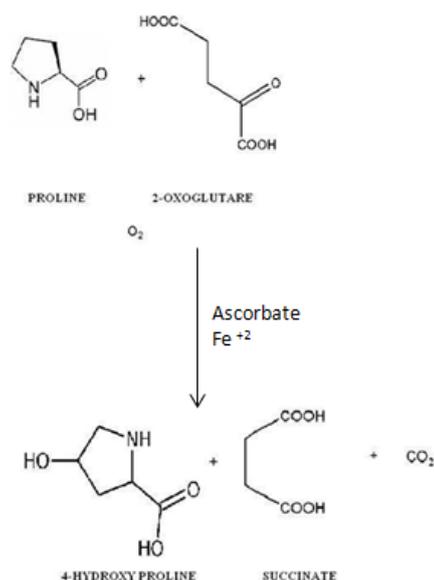


Figure 1 Hydroxylation of prolyl residues to 4-hydroxyproline (E.Jespord, 2005)

Till today, a function similar to HIF-P4Hs has not been identified in plants. Nevertheless, the proteome of a plant cell wall is abundant in hydroxyproline-rich glycoproteins (HRGPs), a superfamily of glycoproteins that play an essential role in almost all aspects of plant growth and development.

Prolyl hydroxylation is significant for plant growth and to this direction may affect plant yield, since leaf growth and the productivity of plant are related. Moreover, ripening excised tomato pericarp discs and carnation petals treated with P4H inhibitors had lower ethylene production, which affected the evolution of ripening and senescence processes respectively (Fragkostefanakis et al., 2011 unpublished data; Vlad et al., 2010). Collectively, these results emphasize the importance of P4Hs in plant developmental processes. This study aimed to analyze the effect of P4H3 and P4H8 on tomato plant growth and its organs and their organs growth correlations due to silencing.

2. METHODS

Today, the vast amount of information emerging from the sequencing of genomes from different organisms strengthens the necessity for the development of more rapid and reliable reverse genetics techniques. Virus-

induced gene silencing (VIGS) is a reverse genetics tool used for the analysis of gene functions. VIGS technology uses viral vectors which carry a fragment from a gene of interest in order to produce dsRNA which eventually triggers RNA-mediated gene silencing [4]. Since gene expression is suppressed at the posttranscriptional level, VIGS, among other RNAi techniques, belongs to the post-transcriptional gene silencing (PTGS) methods such as quelling in fungi [5] and RNA interference in animals [6]. VIGS allows the identification of a loss-of-function phenotype for a specific gene within a single generation because the gene of interest is directly targeted in VIGS, in contrast with technologies such as chemical mutagenesis, transposons or T-DNA insertions [7]. In general, the phenotype due to VIGS-silencing might be developed from 10 to 15 days after the delivery of the virus vector into the plant cells ([8]. VIGS has been used for the functional characterization of genes in different plant organs such as roots [9], leaves [10], tubers, flowers [11] and tomato fruits [12]. The relative expression of P4H3 and P4H8 was determined in control and VIGS plants by real-time PCR. Total RNA was isolated from the pooled tissues of 19 plants and subjected to RNase-free DNase treatment. Real-time PCR was performed using SYBR Green on the ABI PRISM 5700 sequence-detection system. Morphological analysis was performed for P4H3-, P4H8-VIGS and control tomato plants. Nineteen plants were used from each treatment. Six weeks after the infection, the plants were photographed, and then their shoot, root length, fresh weights and leaf areas were determined individually. The area of the 3rd, 4th, 5th and 6th leaf of P4H3-, P4H8-VIGS and control plants was measured using the online leaf area assessment tool (OLA) (13).

Statistical Analyzes

The experimental design was a completely randomized. Data were subjected to T-test analysis for paired samples with significant difference at $p < 0.05$, using the statistical software SPSS Statistics for Windows. Furthermore the effect of VIGS application in different plant organs such as root, shoot, root fresh weight, shoot fresh weight, leaves area were analyzed. Linear correlation was measured by calculating the Pearson correlation coefficient in order to see if there are any correlations between these

variables using Statistical software SPSS Statistics for Windows.

3. RESULT

The expression profiles of P4H3 and P4H8 were monitored in VIGS- treated and control plants, six weeks after virus infection by quantitative real-time PCR. The expression of each gene is presented as relative to the expression in the control plants. VIGS-Silencing resulted in partial suppression of expression of P4H3 and P4H8 by 59% and 64%, respectively, compared to the expression in the control plants in several biological replicates.

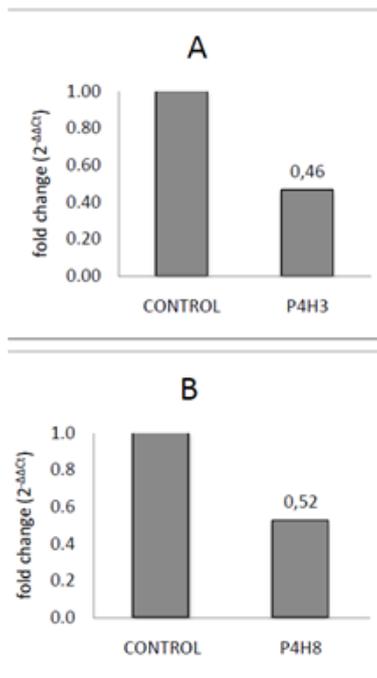


Figure 2 relative expressions of P4H3 and P4H8 in control VIGS-treated plants. Relative fold changes were calculated based on the comparative Ct method, using actin as an internal standard. The Ct value for each gene was normalized to the Ct value for actin and was calculated relative to a calibrator (control) using the formula $2^{-\Delta\Delta C_t}$. Vertical bars are the average of three replications.

The shoots were shorter in VIGS-treated plants of both genes $p > 0,05 P = 2,36E-07$ (Fig3). The average shoot length of P4H3- and P4H8-VIGS treated tomato plants was 13.4 and 16.5cm, respectively while the shoot of control plants was 19cm long (Fig. 3.14). On the other hand, no significant differences were observed in the root length of the control and VIGS-treated plants. P4H3- and P4H8-VIGS treated plants had shorter stems but no effect on root length. The VIGS-P4H3 and P4H8 plants 3th, 4th and 5th leaves are smaller than

the control plants while 6th leaves are not different in size.

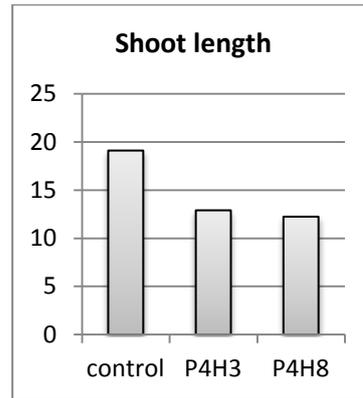


Figure 3 There are significant differences on shoot lengths between control and P4H's group. $p > 0,05 P = 2,36E-07$

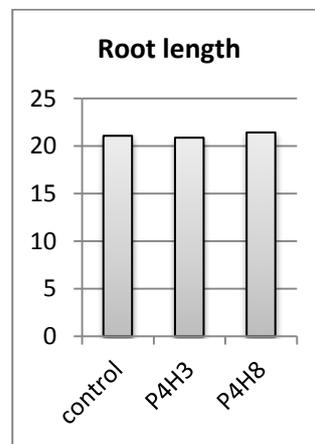


Figure 4 There are no significant differences on root length between control and P4H's group. $p > 0,05, P = ,91$

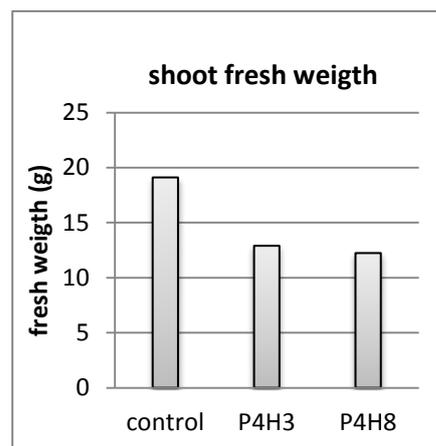


Figure 5 There is significant differences on shoot fresh weight between control and P4H's groups. $P = 1,55E-05, p > 0.05$

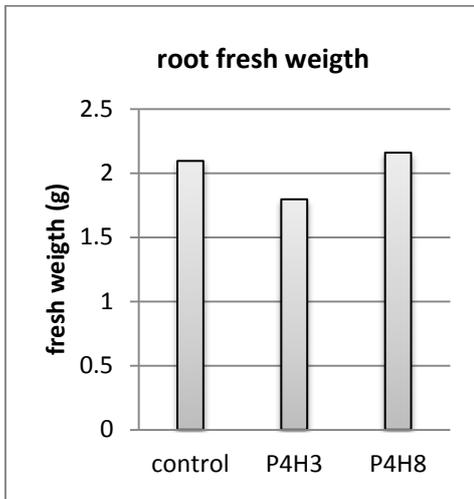


Figure 6 There are no significant differences on root fresh weights between control and P4H's groups. $P=0,123$, $p>0.05$

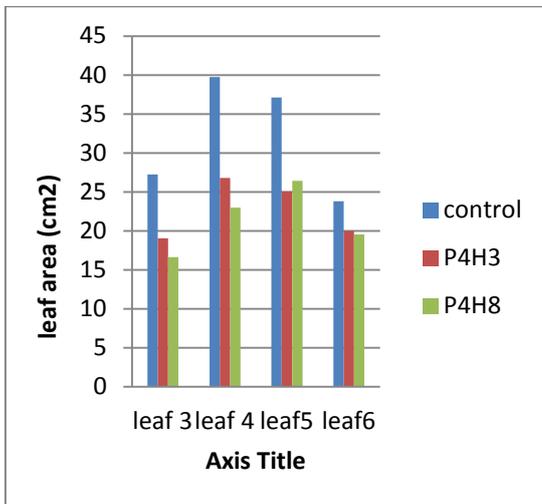


Figure 7 There is significant differences on 3th, 4th, 5th but no differences in leaf 6 ($P= 1,96E-08$, $2,09E-08$, $0,000214$, $0,217577$ respectively at $p>0.05$).



Figure 8 Representative six-week-old control-, P4H3 and P4H8 VIGS-treated plants.



Figure 9 Root length of six-week-old control P4H3-, and P4H8-VIGS.

The area of the 3rd, 4th, 5th and 6th leaf of the P4H3- and P4H8-VIGS treated plants was determined and compared to the respective leaf areas of control plants. The 4th, 5th and 6th leaves of both P4H3- and P4H8-VIGS plants had a significantly smaller area than the leaves of the control plants. In particular, the area of the 3th, 4th, 5th and leaves of the P4H3 plants was 28%, 27% and 23% smaller than the control plants. On the other hand, while the and 6th leaf of the P4H3 and P4H8-VIGS plants were similar to the control plants, the 4th and 5th leaves were 23% and 17% smaller, respectively in P4H8-VIGS.

Correlation between shoot length, root length, stem fresh weight, root weight and all tree leaves were analyses and the results indicated in the following table.

Correlations in Control, P4H3, P4H8								
Organ s of plants	Shoot lenght	Root lenght	Shoot fresh weight	Root fresh weight	Leaf 3	Leaf 4	Leaf 5	Leaf 6
Shoot lenght	1	-	-	0,463	-	-	-	-
	1	0,529	-	0,468	-	0,553	0,457	0,615*
	1	-	-	-	0,515	-	-	-
Root lenght	1	-	-	-	-	-	0,486	-
	1	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-
Shoot fresh weight	1	-	-	-	-	-	-	-
	1	0,820*	-	0,731*	0,597*	0,579*	-	-
	1	-	-	-	-	-	-	-
Root fresh weight	1	-	-	-	-	0,753*	-	-
	1	-	-	0,690*	0,558	0,468	-	-
	1	-	-	-	-	-	-	-
Leaf 3	1	-	-	-	0,522	-	-	-
	1	-	-	0,595*	-	-	0,543	-
	1	-	-	-	-	-	-	-
Leaf 4	1	-	-	-	0,651	0,579*	-	-
	1	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-
Leaf 5	1	-	-	-	-	-	0,563	-
	1	-	-	-	-	-	0,746*	-
	1	-	-	-	-	-	0,623*	-
Leaf 6	1	-	-	-	-	-	-	1
	1	-	-	-	-	-	-	1
	1	-	-	-	-	-	-	1

Table 1The indicated numbers represent the correlation between different organs at $p < 0.05$. (*) represents the correlation at $p < 0.01$. Each row in each cell represent correlation within Control, P4H3, P4H8, respectively.

4. DISCUSSION AND CONCLUSION

Prolyl hydroxylase is important for plant growth and development. Leaf size is of particular interest for tomato crop, since along with stem density and number of leaves per stem influence light interception and subsequently photosynthesis and tomato yield. To this direction, we investigated the role of two P4Hs, P4H3 and P4H8 using a reverse genetics approach such as VIGS. P4H3- and P4H8-VIGS treated plants had shorter stems but no effect on root length. The VIGS-P4H3 and P4H8 plants 3th, 4th and 5th leaves are smaller than the control plants while 6th leaves are not different in size. In all groups there are different correlations between leaves as expected, especially in VIGS-P4H3 there is correlations between leaves in all VIGS plants except between leaf 3 and leaf 5. Although in control groups, in most of the organs there is no correlations that could be biologically significant related to plant growth, there is significant correlation between different organs in P4H3 silenced plants. There is also high correlation between shoot weight and all leaves and root weight and all leaves in VIGS-P4H plants while no correlations seen in the control group. This result shows us that the viral replication occurred in every organs on plants and VIGS was successfully applied. Although VIGS-P4H8 plants have very similar morphological changes with P4H3, interestingly, their organs size changes are not correlated to each other as it is in the P4H3. To figure out the reason should be analyzed in the further studies. All these analyze could be concluded that P4H has important role in plant organs growth and these growths are correlated to each other.

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