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Original Article

Global methylation in 'Valencia' orange seedlings associated with rootstocks and Huanglongbing

Metilação global em mudas de laranja valência associadas a porta-enxertos e ao Huanglongbing

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Abstract

Citrus farming is one of the main activities that contributed to the Brazilian trade balance, with citrus seedling being the most important input in the formation of orchards to guarantee high productivity and fruit quality, which fundamentally depends on the chosen genetics. The present study aimed to analyze the existence of epigenetic variability in 'Valencia' orange plants on rootstocks, associated or not with HLB, through the quantification of the global methylation of its genome, in order to support works on genetic improvement and crop production. For this purpose, this work was carried out in greenhouse in a completely randomized experimental design, with 5 treatments and 6 replicates per treatment, each seedling being considered a replicate, namely: T1 = "Valencia" orange grafted onto "Rangpur" lemon, inoculated with HLB; T2 = "Valencia" orange grafted onto "Swingle" citrumelo, inoculated with HLB; T3 = "Valencia" orange grafted onto "Rangpur" lemon, without HLB inoculation ; T4 = "Valencia" orange grafted onto "Swingle" citrumelo, without HLB inoculation ; T5 = "Valencia" orange in free standing. The DNA was extracted from leaves and the ELISA test (Enzyme-Linked Immunosorbent Assay) was carried out, based on the use of receptors sensitive to 5-mC., to measure the relative quantification of global methylation between genomic orange DNAs . Since the control treatment (T5) consists of "Valencia" orange in free standing, it could be inferred that both the normal grafting technique in the seedling formation process and the inoculation of buds infected with HLB are external factors capable of changing the methylation pattern in the evaluated plants, including the DNA demethylation process, causing an adaptive response in association with the expression of genes previously silenced by genome methylation.

Keywords: epigenetics, citrus culture, phenotypic plasticity.

Resumo

A citricultura é uma das principais atividades econômicas contribuintes à balança comercial brasileira, sendo a muda cítrica o insumo mais importante na formação de um pomar para a garantia de uma boa produtividade e qualidade dos frutos, a qual depende fundamentalmente da genética escolhida. O presente teve como objetivo analisar a existência de variabilidade epigenética em plantas de laranja Valência sobre porta-enxertos, associadas ou não ao HLB, por meio da quantificação da metilação global de seu genoma, a fim de subsidiar trabalhos de melhoramento genético e produção da cultura. Para tanto, o trabalho foi desenvolvido em casa de vegetação, em delineamento experimental inteiramente casualizado, com 5 tratamentos e 6 repetições por tratamento, sendo cada muda considerada uma repetição, sendo eles: T1 = laranja "Valência" enxertada sobre limão "Cravo", inoculada com HLB; T2 = laranja "Valência" enxertada sobre citrumelo "Swingle", inoculada com HLB; T3 = laranja "Valência" enxertada sobre limão "Cravo", sem inoculação com HLB; T4 = laranja "Valência" enxertada sobre citrumelo "Swingle", sem inoculação com HLB; T5 = laranja "Valência" em pé franco. Realizou-se a extração do DNA das folhas e em seguida realizou-se o teste de ELISA (Enzyme-Linked Immunosorbent Assay), baseada no uso de anticorpos sensíveis à 5-mC., para mensurar a quantificação relativa de metilação global entre os DNAs genômicos da laranja. Uma vez que o tratamento controle (T5) consta da laranja "Valência" em pé franco, pode-se verificar que tanto a técnica de enxertia normal no processo de formação de mudas, quanto a inoculação de borbulhas infectadas com HLB são fatores externos capazes de alterar o padrão de metilação nas plantas avaliadas, inclusive, no processo de desmetilação do DNA, provocando uma resposta adaptativa em associação a expressão de genes antes silenciados pela metilação do genoma.

Palavras-chave: epigenetica, citricultura, plasticidade fenotípica.

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1. Introduction

Citrus farming stands out as the most important fruit crop in the world, with production of 262.97 million 40.8-kg boxes in the 2021/22 orange harvest in the citrus belt of the state of São Paulo and Triângulo/Southwestern region of Minas Gerais (FUNDECITRUS, 2022). Among Brazilian regions, the Southeastern region accounts for 83.9% of national production (IBGE, 2019), showing high efficiency in the citrus chain from planting and growth to juice production.

However, to guarantee high productivity and fruit quality, it is extremely important to have quality seedlings for the formation of the orchard, which fundamentally depends on the chosen genetics, combining scion and rootstock varieties, which are directly responsible for the vigor of the scion variety, in addition to influencing nutrient absorption, production quality, water deficiency and salinity, and tolerance to diseases and pests (Schafer et al., 2001).

Diseases and pests can occur in a region and, in a few years, they can spread and have great economic impact (Sun et al., 2019). Various approaches, such as the use of chemical pesticides and other synthetic molecules, have been used to control these problems in cultivated plants, including citrus fruits (Prabha et al., 2017). Huanglongbing (HLB) is considered the main citrus disease in the world and is threatening the sustainability of the citrus activity in affected regions due to its rapid spread and severity of symptoms (Alquézar et al., 2021).

In this sense, the phenotypic plasticity of plants is an important tool for the survival of the species and adaptation to biotic and abiotic exposures (Dooren et al., 2020), and can be used in the genetic improvement of plants to bring benefits to the crop.

Epigenetics is the part of genetics that deals with heritable modifications in gene expression not associated with changes in the gene sequence itself (Jablonka and Raz, 2009). It is also about implications in its structure and accessibility in transcription, without involving mutations or modifications in the sequence of DNA bases (Casadesus and Low, 2006; Bird, 2007), and methylation is the most known epigenetic mechanism, frequently being associated with gene silencing, genome stability, genomic imprinting and transcription repression (Zhang et al., 2018).

Thus, studies have shown some epigenetic modifications that affect important agronomic traits (Manning et al., 2006; Hauben et al., 2009; Martin et al., 2009), and the ability to detect and quantify DNA methylation efficiently and with greater precision demonstrates the importance of this area for science in agriculture for the varietal and behavioral differentiation of plants.

In view of the above, the aim of this study was to analyze the existence of epigenetic variability in 'Valencia' orange plants on rootstocks, associated or not with HLB, through the quantification of global methylation of its genome in order to support works on genetic improvement and crop production.

2. Material and Methods

The experiment was carried out at the Faculty of Agricultural and Technological Sciences, Dracena Campus, FCAT/UNESP, located in the municipality of Dracena (SP), whose geographic coordinates are 21°28'57" S latitude and 51°31'58" W longitude and average altitude of 421m a.s.l., with subtropical cwa regional climate (mild and dry winters followed by very hot summers), according to the Köeppen classification (Köeppen, 1948), and average annual temperature of 23.6° C.

"Valencia" orange seedlings were grafted onto "Rangpur" Lemon and "Swingle" citrumelo rootstocks, inoculated or not with buds contaminated with the huanglongbing bacteria, a procedure carried out in a controlled environment at the Citrus Defense Fund (FUNDECITRUS), municipality of Araraquara - SP, following its safety protocols, distributed in a completely randomized design, containing 5 treatments and 6 replicates per treatment, each seedling being considered a replicate, totaling 30 seedlings.

Treatments used are described as follows: T1 = "Valencia" orange grafted onto "Rangpur" lemon, inoculated with HLB; T2 = "Valencia" orange grafted onto "Swingle" citrumelo, inoculated with HLB; T3 = "Valencia" orange grafted onto "Rangpur" lemon, without HLB inoculation; T4 = "Valencia" orange grafted on "Swingle" citrumelo, without HLB inoculation; T5 = "Valencia" orange in free standing.

The genomic material used was extracted from seedling leaves, collected after 120 days after the inoculation with buds contaminated with the huanglongbing bacteria, using the methodology of Lodhi et al. (1994), and the resulting DNAs were quantified with the aid of NanoDrop 2000 - Thermo Scientific spectrophotometer for further methylation analysis.

The ELISA test (Enzyme-Linked Immunosorbent Assay), a methodology essentially based on the use of antibodies sensitive to 5-mC., was used to perform the relative quantification of global methylation between genomic DNAs. For this procedure, the Imprint DNA Methylation Quantification kit (Sigma) was used.

The absorbance of the solution contained in wells was performed at wavelength of 450nm in a PowerWav XS Microplate Reader (Biotek) equipment. After reading the crude values, the formula used to obtain the corrected values was: [(A450 Sample - A450 Blank)/ A450 control Methylated DNA - A450 Blank)]X100.

After this procedure, performed in duplicate, differences in methylated DNA between cultivars were evaluated with Analysis of Variance (ANOVA) and the SISVAR software (Ferreira, 2019) in order to verify the statistical significance between the different values. *Post-hoc* analyses were performed using the Tukey's test at 95% reliability.

3. Results and Discussion

Table 1 shows absolute and relative quantifications of the global methylation content in the genome in analyzed citrus plants, where it could be observed that there was statistical difference between them.

According to the analysis of variance, there is statistical difference in the percentage of methylated DNA between treatments, and the control (T5) treatment presented global

Table 1. DNA absorbance values of "Valencia" orange seedlings grafted on rootstocks, associated or not with HLB, obtained by the ELISA test with anti 5-mC antibodies and their respective analysis of variance. Dracena, SP, Brazil, 2021.

Source of variation	Absorbance 450nm	Relative quantification of methylated DNA (%)
	First analysis	
Treatments	Mean Square	
	0.018730*	
1	0.251b	44.00
2	0.206c	36.42
3	0.246b	43.50
4	0.217c	38.30
5	0.453a	80.00
Mean	0.275	-
CV %	3,94	-

*Significant by the Tukey's test at 5% probability. Different letters in the column differ statistically from each other. CV = coefficient of variation.

methylation level higher than the other treatments, with 80% of its genome methylated.

Rodrigues et al. (2022), working with the characterization of fig accessions by analyzing the natural root-knot nematode and leaf rust incidence in relation to the epigenomic profile of the plants, concluded that methylation and leaf rust incidence were correlated when observed in the same phonological phase, presenting initial evidence of the same factorial pressure loads in genotypes, suggesting similar behavior within these genotypes. In plants, DNA methylation is used for transposable element (TE) repression, gene expression regulation, and developmental regulation. TE activity strongly influences genome size and evolution, making DNA methylation a key component in understanding divergence in genome evolution (Mbichi et al., 2020). In addition, it can increase variations of quantitative traits because many genes can be affected simultaneously (Phillips et al., 1990).

However, there is the process opposite to this event, known as DNA demethylation, which can be defined as the process reverse to methylation and is also a reversible process (Ponferrada-Marín et al., 2010).

DNA demethylation can occur through two processes: active, which requires enzymatic action and results in the removal of the CH_3 radical, or passive, triggered by the loss of the methyl radical due to inhibition or absence of maintenance DNA methyltransferase (DNMT) (Patel et al., 2010).

Thus, since the control treatment (T5) consists of "Valencia" orange in free standing, it could be observed that both the normal grafting technique in the seedling formation process (Treatments 1 and 3) and the inoculation of buds infected with HLB (Treatments 2 and 4) are external factors capable of altering the methylation pattern of evaluated plants, including the DNA demethylation process (Figure 1).

Furthermore, it was observed that treatments whose plants were inoculated with HLB revealed an even lower methylation content when compared to both control and grafted but uninfected treatments due to a possible influence on plant immunity (Figure 1).

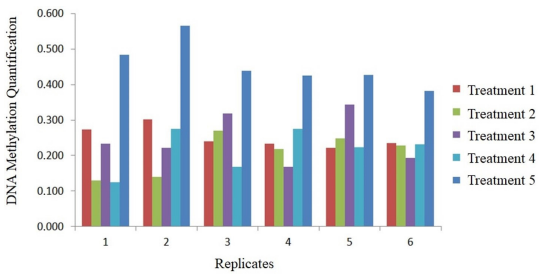

Figure 1. Data dispersion of DNA absorbance values of "Valencia" orange seedlings grafted on rootstocks, by replicates, associated or not with HLB, obtained by the ELISA test with anti 5-mC antibodies and their respective analysis of variance. Dracena, SP, Brazil, 2021.

Zhang et al. (2006) reveal that while DNA methylation can be established and maintained, DNA demethylation also occurs in plants and animals, when methylation pathways are inactivated, DNA methylation is diluted after DNA replication, leading to passive DNA demethylation.

In other cases; however, DNA methylation is removed by active DNA demethylation pathways. Active or passive DNA demethylation can simultaneously reduce DNA methylation during some specific stages of development (Oliveira et al., 2010).

According to Morgan et al. (2004), active demethylation involves demethylases and seems to be necessary to activate specific genes or erase the epigenetic mark during development or in response to environmental disturbances.

Barreto et al. (2007) worked with a nuclear protein involved in maintaining genome stability and concluded that its expression is directly linked to active DNA demethylation, activating silenced genes.

Studies on methylation in the development of mutant *Arabidopsis* seeds obtained evidence of loss of methylation at CG sites in the endosperm (Gehring et al., 2009; Hsieh et al., 2009). Collectively, these studies suggest that mutation is involved in active demethylation of the maternal genome, which gives rise to the endosperm, resulting in

increased siRNA production in this tissue (Mosher et al., 2009; Mosher and Melnyk, 2010).

According to Boyko and Kovalchuk (2010), regulation of gene expression measured by siRNA plays a key role in development and physiological and stress-related processes in plants. Furthermore, the existence of a stress-induced set supports the possible involvement of these siRNAs in the establishment of epigenetic marks, since in plants, epigenetic regulation is responsible for their responses to the environment, such as biotic and abiotic stresses (Pecinka et al., 2010; Hauser et al., 2011).

Thus, DNA demethylation in plants seems to be associated with a possible response to stress, demonstrating that both the grafting process itself and exposure to pathogenic agents trigger the process as a response to their adaptation to that environment.

It could be concluded that, based on results of DNA demethylation of grafted plants in relation to the "Valencia" cultivar in free standing, apparently, both the grafting process and the exposure of plants to HLB cause an adaptive response in association with the expression of genes previously silenced by genome methylation.

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