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A paraquat-inducible protein B-like (*pqiB*) gene from *Chromobacterium violaceum* confers tolerance to paraquat in transgenic tobacco

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Genet. Mol. Res. 23 (1): gmr19176
Received August 07, 2023
Accepted February 21, 2024
Published March 13, 2024
DOI http://dx.doi.org/10.4238/gmr19176

ABSTRACT. The *Chromobacterium violaceum pqiB* gene can be functionally expressed in plant cells and used for the development of paraquat-tolerant crops. Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a contact non-selective herbicide, widely used in agriculture in several countries. Proteins induced by paraquat have been the subject of great interest because of the possibility of conferring herbicide resistance when introduced into crops. In this work, we analyzed a paraquat-inducible B-like protein (*cvpqiB*) gene, isolated from *Chromobacterium violaceum*, for its capacity to confer tolerance to paraquat in transgenic tobacco. A DNA fragment containing the *pqiB* coding sequence was isolated from the *C. violaceum* ATCC12472 genome, inserted into the pCAMBIA1390 vector, under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and used in *Agrobacterium*-mediated transformation of *Nicotiana tabacum* cv. Havana. Analysis of the regenerants revealed the incorporation of *cvpqiB* into the tobacco genome and its transmission in a Mendelian fashion to the progeny of transgenic plants. Sensitivity assays using tobacco leaves demonstrated that the transgenic plants were tolerant to paraquat with concentrations up to 50 µM, whereas the wild-type (WT) plants exhibited intolerance to concentrations higher than n1 µM of the herbicide. Paraquat-treated leaves of the transgenic plants also exhibited significantly reduced electrolyte leakage and their chlorophyll content was not impacted as observed in the WT plants. Besides, in contrast to

the WT, negligible amounts of hydrogen peroxide were detected in paraquat-treated seedlings of the transgenic plants, as revealed by 3,3'-diaminobenzidine staining. Collectively, these results indicate that the *cvpqiB* gene is functional in plants and may be further used in the genetic engineering of crop plants aiming at paraquat tolerance.

Key words: Herbicide; Crop development; Hydrogen peroxide; Plant resistance

INTRODUCTION

Herbicides are frequently used in agriculture to facilitate the management of invasive plants without causing damage to crops. Herbicide resistance is a relevant feature in cultivated plants, and it can be an intrinsic feature to the plant or induced by changes at a molecular level (Dodge, 1989). Genetically transformed plants with features that confer resistance to herbicides have been produced and largely used in agriculture in recent decades (Heap, 2014; Majumder et al., 2024).

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a contact non-selective herbicide widely used in agriculture in many countries, as it is low-cost, highly effective, and has low cumulative pollutant effects on the soil (Bromilow, 2004). It is known that paraquat increases the production of superoxide radicals that have a detrimental effect on photosynthesis, diverting electrons from the photosystem I (PSI) iron-sulfur centers in chloroplasts, generating hydrogen peroxide radicals and causing cell membrane disintegration and ion leakage (Dodge, 1989; Dayan et al., 2015). Inhibition of electron flow in PSI also damages the PSII due to a loss of functional capacity of the major carries between photosystems, which leads to the accumulation of electrons and imbalance of the proton gradient between the chloroplast stroma and lumen. Reactive species produced by re-oxidation of reduced mparaquat reach the reaction center of PSII and cause a further loss of photosynthetic efficiency (Bromilow, 2004; Hawkes, 2013).

The efficiency of antioxidant enzymes, such as copper zinc superoxide dismutase (CuZnSOD), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR), in improving tolerance to paraquat has been previously demonstrated (Iannelli et al. 1999; Lee et al., 2007). Genes encoding paraquat-inducible protein, such as the *Ochrobactrum anthropi pqrA* (Jo et al., 2004), as well as anti-apoptotic protein, such as the human *Bcl-xL* (Chu et al. 2008) have been isolated and also demonstrated to confer tolerance to paraquat when expressed in plant cells.

In this study, we performed a functional analysis of a *pqiB*-like (*cvpqiB*) gene isolated from *Chromobacterium violaceum*, a Gram-negative proteobacterium first described at the end of the 19th century and that dominates a variety of ecosystems in tropical and subtropical regions. Different lineages of *cvpqiB*-overexpressing transgenic tobacco plants were generated and submitted to paraquat treatments. To the best of our knowledge, this is the first time that a gene encoding paraquat-inducible protein B (PqiB) isolated from *C. violaceum* is tested in plant cells.

MATERIAL AND METHODS

Sequence Analysis

The *cvpqiB*-like gene sequence was retrieved from the complete genome sequence of *C. violaceum*, obtained by the Brazilian National Genome Project Consortium (Vasconcelos et al., 2003). This open reading frame (ORF) was identified as CV2547 (Vasconcelos et al., 2003). The CvpqiB amino acid sequence and properties were analyzed by the tools available at Expasy Derver (http://web.expasy.org) (Gasteiger et al., 2005). The transmembrane helices and domain families were predicted by the TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and InterPro 52.0 (http://www.ebi.ac.uk/interpro/) (Sonnhammer et al., 1998; Hirokawa et al., 1998; Mitchel et al., 2015).

Vector construction and *Agrobacterium* strain

The molecular cloning procedures were performed as described by Sambrook et al. (1989). The *cvpqiB*-like coding sequence was amplified from the *C. violaceum* genome (ATCC12472 strain) by PCR with primers containing restriction sites for *Eco*RI. The forward primer was 5'-CCGGTAGAGGAATTCGATGAGC-3' and the reverse primer was 5'-CGGGAATTCAATGTCTGATCATGG-3'. The 1,654 kb amplified fragment was digested with *Eco*RI and cloned into the same restriction site of a modified pCAMBIA 1390 binary vector. The resulting construction contained the *cvpqiB*-like gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (*nos*) terminator, as well as the hygromycin phosphotransferase II (*hptII*) gene under the control of the CaMV 35S promoter (Electronic Supplementary Material - ESM Figure S1). This construction was introduced into *Agrobacterium tumefaciens* EHA105 by direct DNA uptake.

Nicotiana tabacum transformation via *Agrobacterium tumefaciens*

Leaf discs of *Nicotiana tabacum* cv. Havana were used in the *Agrobacterium*-mediated genetic transformation experiments. The genetic transformation procedures were performed as previously described by da Silva et al. (2011). Briefly, the leaf discs were cultivated for 15 min with *A. tumefaciens* solution, washed with autoclaved water and then placed on Petri dishes containing MS medium (Murashige and Skoog, 1962) supplemented with sucrose (60 g L^{-1}), 6-benzylaminopurine (500 mg L^{-1}), cefotaxime (400 mg L^{-1}) and hygromycin sulfate (40 mg L^{-1}). After one month, the regenerated shoots were isolated and rooted on an antibiotic and plant hormone free MS medium. Twenty different plants, representing distinct transformation events, were selected, transferred to soil and grown in greenhouse, under controlled conditions. Each plant represents a transgenic lineage (P1 to P20). Two wild-type (WT, not transformed) plants were used as controls. We selected the 5 most vigorous transgenic lineage to perform further analysis (P3, P6, P13, P16 and P20).

Molecular analysis of the transgenic plants

Genomic DNA was extracted from leaves of transgenic and WT plants using the CTAB method (Doyle and Doyle 1990). The presence of the *hpt*II and *pqiB*-like genes was analyzed by PCR. The primer sequences used for amplification of the *hpt*II gene were 5'-ATGGGGATTGAACAAGATGGATTG-3' and 5'-

TCAGAAGAACTCGTCAAGAAGGC-3', while those for the *cvpqiB*-like gene were described above. PCR reaction was carried out in 25 µL volumes containing 0.25 U *Taq* DNA polymerase (Promega, Madison, USA), 0.2 mM of each dNTP, 0.25 µM of each primer and 50 ng of sample DNA. Reaction conditions were 35 cycles of 94^{o}C for 45 s, 62ºC (*hptII*) or 61°C (*cvpqiB*) for 60s and 72ºC for 90s, and a final extension at 72ºC for 7 min. Amplified products were resolved on 1% agarose gel supplemented with ethidium bromide (0.1%) and visualized under UV light.

Segregation analysis

Resistance to hygromycin segregation was analyzed in the T_1 progeny obtained by self-pollination by culturing the sterilized seeds on plates containing MS medium supplemented with 40 mg L^{-1} hygromycin. Seeds of the WT plants were used as a control. The plates were kept under controlled conditions (25 ± 2^{o}C, 16-h photoperiod and photon flux density of 40 µmol m^2 s^{-1}) for 15 days before assessment of the frequencies of green (antibiotic-resistant) and white (antibiotic-susceptible) seedling.

Paraquat sensitivity assay

Sensitivity assays to paraquat were carried out by dropping 10 µL of the herbicide in different concentrations (0, 1, 10, 20, 50 and 100 µM) along photosynthetically active leaves, in triplicates. Three herbicide concentrations were dropped on each side of the leaf midrib (ESM Figure S2). The leaves were then isolated and maintained on MS solid medium, under a photoperiod of 16h at 25^{o}C and irradiance of 16-19 µmol m^2 s^{-1}. The extent of necrosis was recorded 5 days after the treatments.

Determination of chlorophyll content

To determine the chlorophyll content, three young leaves, completely expanded, (two to six week old) were isolated from one plant representative of each transgenic and WT lines established *in vitro*. Leaves were brushed with paraquat at 10 µM or water (control) and kept on MS medium for 24 h at 25°C under fluorescent light (16-19 µmol $m^{-2}s^{-1}$). The chlorophyll measurement was performed according to Hiscox and Israelsta (1979), with modifications. Three leaf discs were isolated from each leave and immersed in 1mL of CaCO3- saturated DMSO solution at 65ºC and kept in the dark. After 12h, the absorbance was measured spectrophotometrically at 663 and 645 ηm for the absorption spectra of *a* and *b* chlorophylls, respectively, in spectrophotometer (Versamax, software Softimax pro 4.8, Molecular device), using the DMSO extracts. The chlorophyll content was calculated according to equations proposed by Wellburn (1994).

Determination of ion likage

Three photosynthetically active leaves (two to six weeks old) were isolated from transgenic and WT lines, brushed with paraquat (10 µM) and maintained on MS medium for 24 h at 25ºC under continuous fluorescent light (16-19 µmol $m^{-2}s^{-1}$). The electrolyte leakage analysis was performed as described by Mitler et al. (1999), with modifications. Six leaf

discs were immersed in 4 mL of ultrapure water for 3h at room temperature and the conductivity was measured. After 20 min at 95ºC, total conductivity was measured and calculations were performed. Ultrapure water was used to measure the electrolyte leakage using a conductimeter (Quimis, mod. Q795A).

RESULTS

In silico analysis

The *cvpqiB* coding sequence is 1,620 bp long and encodes a protein of 539 amino acids, with a predicted molecular weight of 58.6 kDa and theoretical isoelectric point (pI) of 9.13. A hydrophobic sequence was identified on analyses using the data from SOSSUI (EMS Figure S3). A transmembrane helix from residues 28 to 50 was identified in the sequence. Residues 1 to 27 are localized inside the cell, while residues 51 to 593 are located outside the cell. Three mce (mammalian cell enter) domains were identified at residues 53 to 142, 168 to 230 and 297 to 395. Twenty shoots were regenerated and PCR analysis confirmed the integration of the transgene (ESM Figure S4). The regenerated shoots showed normal development and after acclimatization the transgenic plants showed a normal phenotype as compared to WT. Furthermore, transgenic and control plants flowered simultaneously after four months in greenhouse. A segregation analysis carried out with five transgenic lineages indicated the integration of two loci of the transgene in the genome of four transgenic lines (3, 6, 16 and 20) and one locus of the transgene integrated in the genome of line 13 (ESM Table 1). These data further confirmed the transfer of transgene to the offspring.

Transgenic plants are more tolerant to paraquat

Transgenic plants leaf showed tolerance to concentrations up to 100 µM of paraquat, while chlorosis followed by necrosis was observed in WT plant leaves in concentrations as low as 1 µM of the herbicide (Figure 1). Paraquat tolerance varied in a certain extent depending on the transgenic line tested. For instance, two replicas from the transgenic line P3 exhibited necrosis only at 100 µM while P6, P16 and P20 lines showed necrosis at 50 µM of paraquat (in one or two replicates).

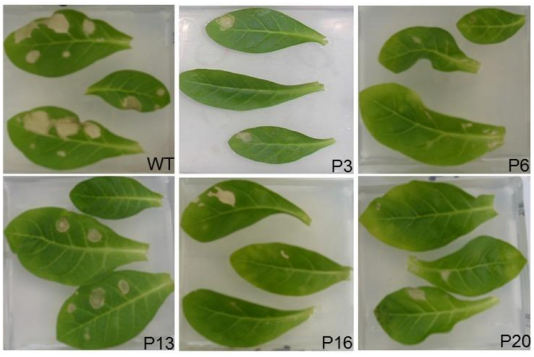

Figure 1. Sensitivity of tobacco plants to different concentrations of paraquat. Three leaves, representative of WT and transgenic lines (P3 – P20), were induced with drops containing 0, 1 and 10 (left side of the leaves, from the base to the apex) and 20, 50 and 100 (right side of the leaves, from the base to the apex) µM paraquat (refer to Material and methods section and ESM Figure S2 for details). Symptoms were recorded 48h after the treaments. WT: wild-type; P3-P20: transgenic lines. Pictures were taken 5 days after the treatments, with three leaf replicates for each plant line tested.

Furthermore, it was observed that in P6 necrose intensity were lower in comparison to all others transgenic lines. The most sensitive transgenic line was P13, which started to developed necrosis at 10 µM paraquat.

Ion leakage and chlorophyll content in paraquat-treated plants

The highest paraquat concentration (10 µM) tolerated by all the transgenic lines tested was used to analyze the ion leakage resistance in their leaves. The results revealed that all transgenic plants presented reduced leakage of ions as compared with WT (Figure 2). These results indicated that the membrane-related damage and loss of the cell content were lower in the transgenic plants. P6, P13, P16, and P20 exhibited differences in ion leakage above 10% in comparison with WT, which was considered significant according to *t*-test ($p \leq 0.01$). Although P3 also showed a lower percentage of ion leakage than the WT, it was not significantly different from the WT.

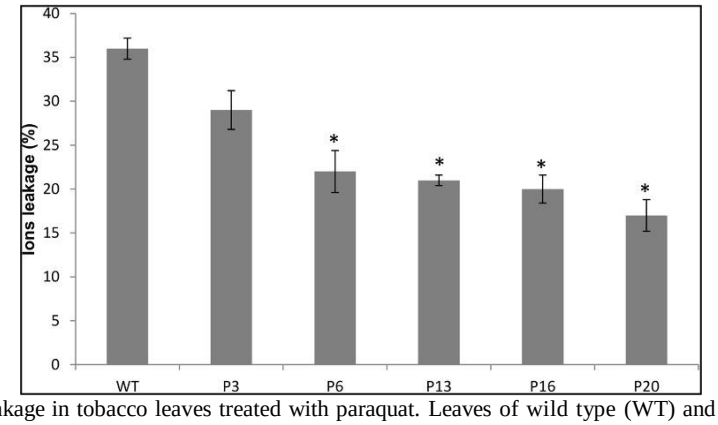

Figure 2. Ion leakage in tobacco leaves treated with paraquat. Leaves of wild type (WT) and transgenic plants were treated with 10 µM paraquat and ion leakage was recorded 24h after the treatment. Data are means ± standard deviation of three biological replicates. *Significant differences according to *t*-test at the $p \leq 0.01$ significance level. WT: wild-type; P3-P20: transgenic line.

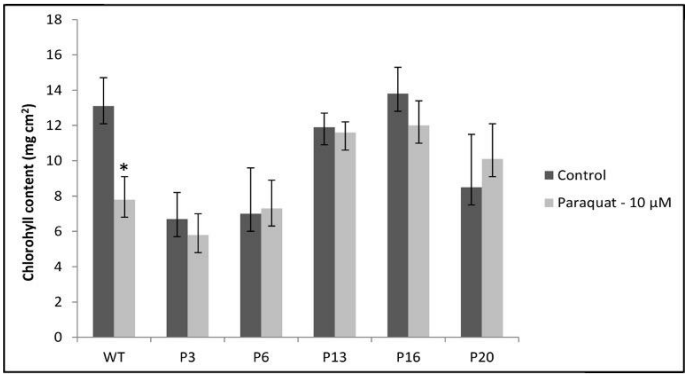

Figure 3. Chlorophyll content of tobacco leaves treated with paraquat. Leaves of WT and transgenic plants were treated with 10 µM paraquat or water (control) and the chlorophyll content was recorded 24 h after treatment. Data are means ± standard deviation of three biological replicates. *Significant differences according to *t*-test at the $p \leq 0.05$ significance level. WT: wild-type; P3-P20: transgenic lines.

Histochemical analysis of H2O2 production

Seedlings of transgenic and WT plants were sprayed with 10 µM paraquat or water and 24 h after treatment they were stained with DAB in order to detect H2O2. It was observed that all seedlings (transgenic or WT) did not stain brown when treated with water (data not shown). However, when treated with paraquat, seedlings of WT were heavily stained throughout and clearly distinct from those of transgenic lines, which were lightly (P6) or not (P3, P13, P16, and P20) stained (Figure 4). These results suggest that the expression of the *C. violaceum pqiB* gene confers protective mechanisms to prevent H2O2 formation or detoxify already formed H2O2. In doing so, the harmful effect of this compound is significantly reduced in transgenic plants treated with paraquat.

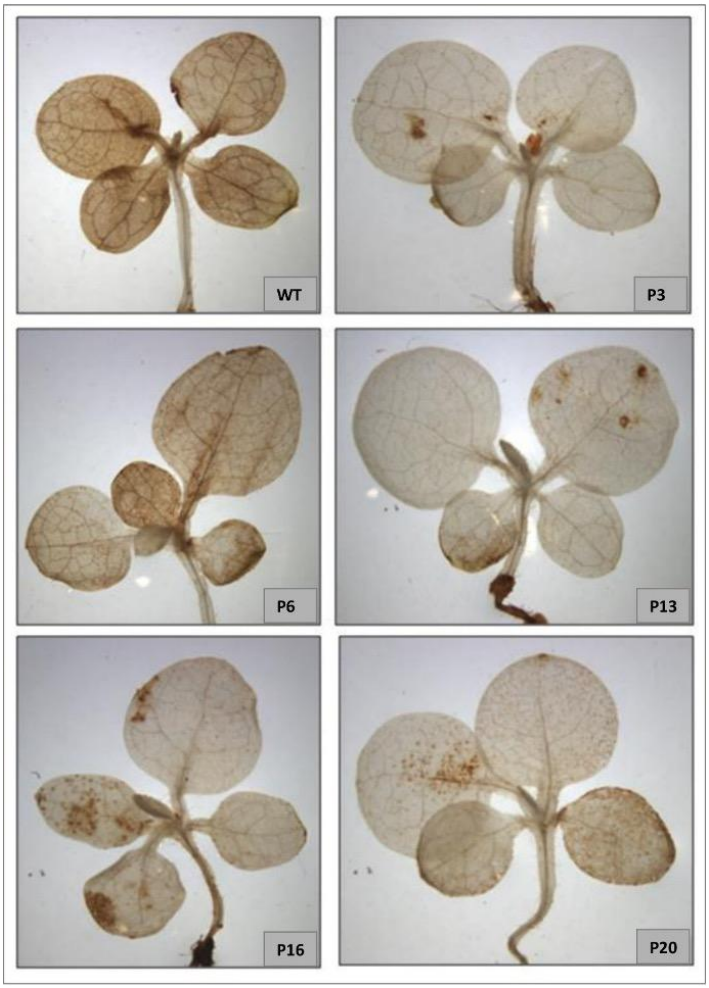

Figure 4. Detection of H2O2 in tobacco seedlings treated with paraquat. Seedlings of WT and transgenic plants were treated with 10 µM paraquat and the H2O2 production, as revealed by reddish-brown stain, was detected by DAB staining, 4 h after the treatments. Figures are representative of experiments performed with 15 replicates from each line. WT: wild-type; P3-P20: transgenic lines.

DISCUSSION

In the present study, transgenic tobacco plants expressing a gene isolated from *C. violaceum*, *cvpqiB,* showed resistance to concentration as high as 10 µM paraquat, with some of them exhibiting resistance to up to 50-100 µM paraquat, while the WT plants were sensitive to 1 µM of the herbicide. Plant resistance to 5-20 µM of paraquat has been considered satisfactory since the herbicide is lethal to most crops and *in vitro* plant cultures in concentrations as low as 5 µM (Iannelli et al. 1999; Lee et al. 2007).

One of the known effects of paraquat is the loss of ions and other solutes in plants after contact with the herbicide (Hawkes 2013; Dayan et al., 2015). This is caused by the capture of electrons generated from the PSI, which induces the formation of H_2O_2, leading to cell membrane peroxidation and damage and, hence, cell death (Murgia et al. 2004; Hawkes 2013). When a plant is resistant to paraquat, these harmful effects of the herbicide are prevented or minimized, allowing the plant to develop in contact with the herbicide. Resistance to paraquat can be acquired by different ways (Hawkes 2013). For example, the pqrA, from *O. anthropic*, and the mvrC, from *E. coli*, are membrane protein that prevent the permeability or enhancing the efflux of paraquat in the cell (Morimyo et al., 1992; Jo et al., 2004) while antioxidant enzymes confer resistance by detoxifying superoxide radicals, like H_2O_2 (Lee et al 2007; Xu et al 2013). The mvrA is a protein identified in *E. coli* that confers resistance to paraquat by similar effects of antioxidant enzymes, reducing toxic species produced by paraquat (Morimyo et al., 1992). According to Cho et al. (2003) the pqrB from *Streptomyces coelicolor* is a membrane protein like efflux bomb that confers resistance to paraquat. We identified three mce domains in this PQIB protein. This transmembrane domain in mammal's cells is related to transport compounds inside or outside the cell. Therefore, the presence of this domain in PQIB suggests that this protein can either prevent paraquat entry inside the cell or it can promote the entry of detoxifying proteins that in turn reduce the paraquat effect. In fact, all transgenic lines tested presented higher tolerance to paraquat, in comparison to WT. The less tolerant transgenic line was able to support up to 10 µM paraquat, while WT plants were sensitive to 1 µM of the herbicide. However, most of the transgenic lines presented tolerance ranging from 50-100 µM of paraquat (Figure 1). Our results revealed that *pqiB* prevents common plant physiological responses to paraquat, such as ion leakage (Figure 2) and reduction of chlorophyll content (Figure 3), indicating that transgenic plants are tolerant when subjected to the same paraquat concentration as the WT. Other transgenic plants resistant to paraquat also showed membrane integrity, low ion leakage, and unaffected chlorophyll content (Mitler et al., 1999; Jo et al., 2004; Kasajima, 2017.

We further observed that paraquat treatment induced less H_2O_2 formation in the transgenic plants compared to the observed in the WT (Figure. 4). These results indicate that *pqiB* plays an important role in providing protection against paraquat-induced oxidative stress. Further functional studies are required to elucidate if the protective mechanism of PqiB involves prevention of H_2O_2 formation or H_2O_2 detoxification. Because paraquat is a potent inducer of oxidative stress and oxidative stress rises as a secondary stress induced by various other environmental stresses, we may further test if these transgenic lines are also tolerant to abiotic stresses (Chu et al., 2008; Seong et al., 2007; Chen and Guo, 2008; Kasajima, 2017).

In conclusion, the *pqiB* gene is able to confer paraquat tolerance in transgenic tobacco and its expression does not affect other phenotypic characteristics of the transgenic plant.

ACKNOWLEDGMENTS

Authors thank "Ministério da Ciência e Tecnologia (MCT)/Conselho Nacional de Desenvolvimento Cientfico e Tecnológico (CNPq), Brazil, for the research funding. We also are grateful for the support of the team and structure of the "Centro de Biotecnologia e Genética (CBG)" of the "Universidade Estadual de Santa da Cruz (UESC)". Authors dedicate the work *in memorium* to Dr Julio Cezar de Mattos Cascardo, who joined the Brazilian National Genome Program (a consortium funded in December 2000 by the MCT through CNPq) for sequencing the *Choromobacterium violaceum* and started coordinating the functional studies with *cvpqi*.

AVAILABILITY OF DATA AND MATERIAL.

The sequence reported in this paper has been deposited in the GenBank database (accession no. AE016825)

AUTHORS CONTRIBUTIONS.

LF was responsible for the execution of all the experimental steps with support from JALS; MGCC and FCA analyzed and discussed the data; LF, MGCC and FCA wrote the manuscript; MGCC and FCA was responsible for the acquired funding of the research and for the advising of LF and JALS.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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