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SHORT COMMUNICATION

CRISPR/Cas9-mediated phospholipase C 2 knock-out tomato plants are more resistant to *Botrytis cinerea*

Enzo A. Perk[1] · Andrés Arruebarrena Di Palma[1,3] · Silvana Colman[1,4] · Oriana Mariani[1,5] · Ignacio Cerrudo[1] · Juan Martín D'Ambrosio[1,6] · Luciana Robuschi[1] · Marina A. Pombo[2] · Hernán G. Rosli[2] · Fernando Villareal[1] · Ana M. Laxalt[1]

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Abstract

Main conclusion **CRISPR/Cas9-mediated Phospholipase C2 knock-out tomato plants are more resistant to *Botrytis cinerea* than wild-type plants, with less ROS and an increase and reduction of (JA) and (SA)-response marker genes, respectively.**

Abstract Genome-editing technologies allow non-transgenic site-specific mutagenesis of crops, offering a viable alternative to traditional breeding methods. In this study we used CRISPR/Cas9 to inactivate the tomato Phospholipase C2 gene (*SlPLC2*). Plant PLC activation is one of the earliest responses triggered by different pathogens regulating plant responses that, depending on the plant–pathogen interaction, result in plant resistance or susceptibility. The tomato (*Solanum lycopersicum*) *PLC* gene family has six members, named from *SlPLC1* to *SlPLC6*. We previously showed that *SlPLC2* transcript levels increased upon xylanase infiltration (fungal elicitor) and that SlPLC2 participates in plant susceptibility to *Botrytis cinerea*. An efficient strategy to control diseases caused by pathogens is to disable susceptibility genes that facilitate infection. We obtained tomato *SlPLC2*-knock-out lines with decreased ROS production upon *B. cinerea* challenge. Since this fungus requires ROS-induced cell death to proliferate, *SlPLC2*-knock-out plants showed an enhanced resistance with smaller necrotic areas and reduced pathogen proliferation. Thus, we obtained *SlPLC2* loss-of-function tomato lines more resistant to *B. cinerea* by means of CRISPR/Cas9 genome editing technology.

Keywords *Botrytis cinerea* · CRISPR/Cas9 · Lipid signaling · Plant defense · Plant resistance · Phospholipase C · *Solanum lycopersicum* · Reactive oxygen species · Transgene-free edited plants

Abbreviations

CRISPR/Cas9	Clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9
Hpi	Hours post-inoculation
JA	Jasmonic acid
PLC	Phospholipase C
SA	Salicylic acid
sgRNA	Single guide RNA
ROS	Reactive oxygen species

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Extended author information available on the last page of the article

Introduction

Genome-editing technologies allow non-transgenic site-specific mutagenesis directly into a crop, offering a viable alternative to traditional breeding methods. *Solanum lycopersicum* is an ideal dicot crop to assay CRISPR/Cas9 given its economic importance. Diseases that affect tomato crops represent significant economic losses and the use of chemicals for their control, apart from having a negative impact on the environment, may lead to resistant generation. Discovering new pathways that can be manipulated is an innovative biotechnological approach for crop protection against pathogens.

Phosphoinositide specific phospholipase C (PLC) is rapidly activated in plant cells after the recognition of different pathogen-associated molecular patterns (PAMPs) and effector proteins (van der Luit et al. 2000; de Jong

et al. 2004; Andersson et al. 2006; Laxalt et al. 2007; Raho et al. 2011). PLC enzymes are encoded by a gene family (Fig. S1) with specific expression and biochemical regulation (Pokotylo et al. 2014). In *Arabidopsis*, we showed that AtPLC2 is required for stomatal immunity during *Pseudomonas* infections and basal resistance against the non-adapted pathogen pea powdery mildew (D'Ambrosio et al. 2017). AtPLC2 interacts with NADPH oxidase D and is required for reactive oxygen species (ROS) production during flagellin perception (D'Ambrosio et al. 2017). This suggests that AtPLC2 plays a role in PAMP-induced immunity (PAMP Triggered Immunity, PTI) and resistance to non-host pathogens involving ROS-regulated processes (D'Ambrosio et al. 2017). The hypothesis is that plants with null or decreased levels of AtPLC2 would be more resistant to pathogens that require ROS for proliferation, such as Botrytis. Tomato genome encodes 6 *PLC* genes, being *SlPLC2* and *SlPLC3* in the same clade as *AtPLC2* (Fig. S1, Clade 2), suggesting similar functions. In tomato, we found that transient *SlPLC2*-silencing resulted in a decreased ROS production and reduced susceptibility to *B. cinerea* (Gonorazky et al. 2016) suggesting that *SlPLC2* is a susceptibility (S) gene that facilitates pathogen infection and proliferation. Disruption of a single S gene is an effective strategy to achieve durable disease resistance in crops. Our goal in this work was to generate *SlPLC2* loss-off-function stable lines by means of the CRISPR/Cas9 genome editing technology to obtain tomato cultivars more resistant to Botrytis.

Materials and methods

Details are provided in Supplementary File S1.

CRISPR/Cas9 vector construction and tomato transformation

All the procedure was performed according to Nekrasov et al. (2017). Briefly, two single guide RNA (sgRNAs) were designed within the first exon (Fig. 1a) in an area that does not show allelic variations using the web page (CRISPR-P 2.0 for plants, http://crispr.hzau.edu.cn/CRISPR2) and cloned into the T-DNA vectors that contain the Cas9 gene optimized for plants (Weber et al. 2011).

Edited *Solanum lycopersicum* MM-Cf0 plants were generated by *Agrobacterium tumefaciens* strain GV3101-mediated transformation according to Van Eck (2019) with modifications (Supplementary File S1). Transformed kanamycin resistant plants were confirmed by amplifying the Cas9 fragment using the primers indicated in Table S1 and gDNA as a template. To assay the DNA edition, PCR products obtained using primers 1 and 2 were sequenced. Then T1 and T2 plants were generated by self-crossing and selected for *SlPLC2* edition (homozygote) by sequencing and for negative Cas9 fragment by PCR.

B. cinerea infection assay

B. cinerea strain B05.10 was maintained and conidia was isolated as described (Benito et al. 1998). Six-week-old tomato leaflets were droplet inoculated with *B. cinerea*.

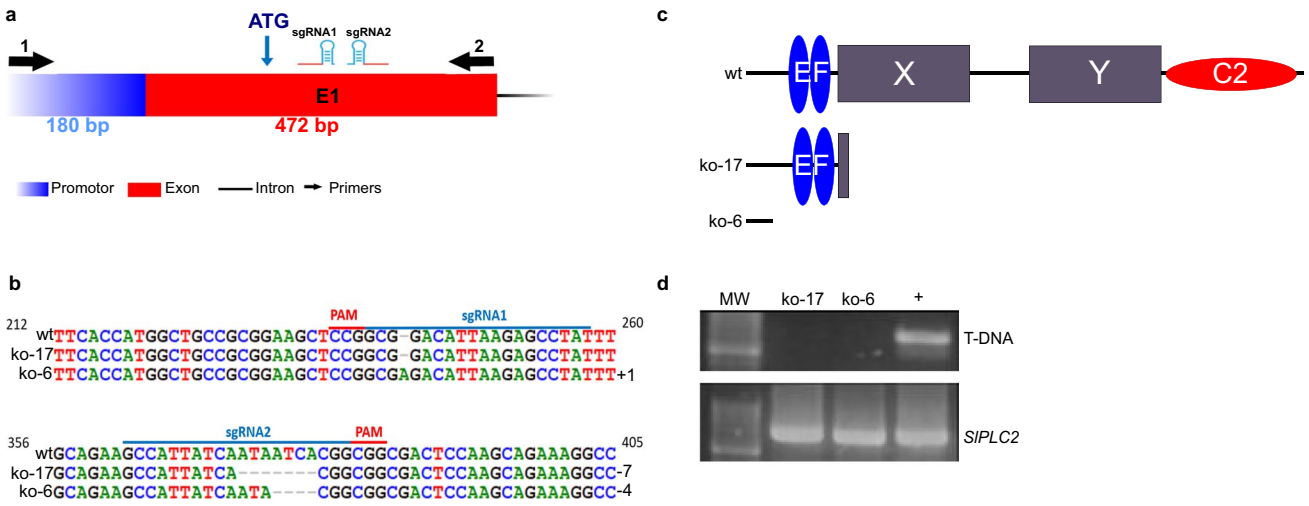

Fig. 1 Targeted editing of *SlPLC2*. **a** sgRNA's design on *PLC2* gene (Solyc06g051620.2). **b** Alignment of nucleotide sequences targeted by sgRNA1 and sgRNA2 of homozygous T1 lines (ko-17 and ko-6). Insertions (+) and deletions (−) are indicated on the right of the sequence. **c** Predicted SlPLC2 proteins (X and Y PLC catalytic domains, EF-hand motifs and C2 domain). **d** Amplification of the Cas9 and *PLC2* of T2 ko-17 and ko-6 gDNAs using as a positive sample gDNA from T0 #6 as control

Per detached leaf, each lateral leaflet was inoculated with 8 droplets of 2 µL (Benito et al. 1998). Lesion diameters of 700–800 inoculation sites were measured with a caliper at 48 and 72 h post-inoculation (hpi) for each line. The average lesion expansion rate was calculated by subtracting to each 72 hpi lesion diameter the corresponding at 48 hpi. Then total area was measured at 72 hpi with ImageJ Software.

Detection of H_2O_2 production in *B. cinerea* infected leaves

B. cinerea droplet inoculated leaflets were harvested at 24 hpi and immediately incubated with 15 mg mL^{-1} 3,3-diaminobenzidine (DAB). DAB locally precipitates as soon as it comes into contact with H_2O_2 in the presence of peroxidase, visualized as a brown precipitate (Thordal-Christensen et al. 1997) and quantified using the program ImageJ and expressed as mean gray value/mm^2.

RT-qPCR gene expression analysis

Total RNA (1 µg) was reverse-transcribed using oligo dT and M-MLV (Invitrogen) reverse transcriptase. RT-qPCR were performed using 1:10 dilution of cDNA template and Fast Universal SYBR Green Master mix from Roche (Mannheim, Germany). Amplified signals were monitored continuously with a Step One Plus Real-Time Thermal Cycler (Applied Biosystem). Nucleotide sequences of the specific primers for qPCR analysis of *SlPLC1-6*, *SlPR1a, SlPI-II, SlACT* and *BcAct* are listed in Table S1 and were previously reported in Gonorazky et al. (2016).

Results

To generate frame-shift *SlPLC2* mutations, we designed two sgRNAs downstream of the ATG codon in the first exon, spaced approximately 50 bp (Fig. 1a, Fig. S2). T0 plants were analyzed by PCR (primer combination 1 and 2) showing in 64 cases a single amplification product in 2% agarose DNA gels, indicating that 50 bp deletions between the two sgRNAs did not occur. Nevertheless, we pursued the analysis by sequencing the PCR fragment of 23 individuals, finding two edited heterozygous lines #6 and #17. We obtained homozygous edited lines screening the T1 offspring by sequencing the PCR fragment (Fig. 1b, Fig. S3). The homozygous lines named ko-6 and ko-17, carried frame-shift mutations with predicted truncated proteins (Fig. 1c, Fig. S3). T2 plants were selected based on absence of the T-DNA (Fig. 1d) and 100% kanamycin susceptibility in germination assays. Both lines did not show any visible phenotypic differences compared to WT (Fig. S4). The transcript levels of the *PLC* gene family showed no significant differences between WT and ko lines (Fig. S5). Thus, we were able to generate two independent Cas9-free homozygous frame-shift *SlPLC2* mutants for functional pathogen resistance assays.

B. cinerea is a necrotroph pathogen that causes gray mold leading to crop losses. We analyzed the role of SlPLC2 during *B. cinerea* infection and found that ko leaflets inoculated with conidia showed smaller necrotic lesions at 72 hpi compared to WT (Fig. 2a). This phenotypic response was quantified finding a significant decrease in lesion spreading between 48 and 72 hpi and the necrotic area at 72 hpi in ko lines (Fig. 2b and c). *B. cinerea* actin transcript levels were significantly reduced in ko lines (Fig. 2d). Altogether *SlPLC2* ko plants showed more resistance to *B. cinerea*. To test if the response is pathogen lifestyle specific, we challenged the ko lines with the hemi-biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000, the causal agent of tomato speck disease. We found no differences in susceptibility compared to WT (Fig. S6).

During pathogen perception, plants release high amounts of ROS that results in local cell death to block pathogen colonization and expression of defense-related genes. To penetrate and grow on the plant, *B. cinerea* actively triggers plant ROS production and cell death during the early infection state leading to fungal biomass accumulation prior to the transition to a late infection phase (van Kan 2006). H_2O_2 production detected by DAB staining 24 hpi was reduced in ko lines (Fig. 2e and f). We also found that transcript abundance of SA-defense-related *SlPR1* gene was lower during *B. cinerea* infections (Fig. 2g), and the JA-defense gene *SlPI-II* expression was higher in ko infected plants (Fig. 1h). *SlPR1* and *SlPI-II* levels in non-infected ko plants were not different than WT (Fig. S7). As reported in Gonorazky et al. (2016), *SlPLC2* gene expression is induced during *B. cinerea* infections. Knock out plants showed less *SlPLC2* transcript levels compared to WT, probably due to mRNAs degradation via non-sense-mediated decay mechanisms of transcript carrying premature stop codons (Fig. 2i).

Discussion

By virus induced-gene silencing we previously showed that SlPLC2 participates in plant susceptibility to *B. cinerea* in tomato (Gonorazky et al. 2016). An efficient strategy to control diseases caused by pathogens is to disable S genes (susceptibility genes that facilitate infection) through gene editing. Now using CRISPR/Cas9 we generated tomato stable plants, transgene-free, loss-of-function *SlPLC2,* by producing deletions using specific sgRNAs that resulted in a frame-shift. These lines have improved resistance to the necrotrophic fungus *B. cinerea*. In addition, we studied the mechanism and find out that knock-out plants are more

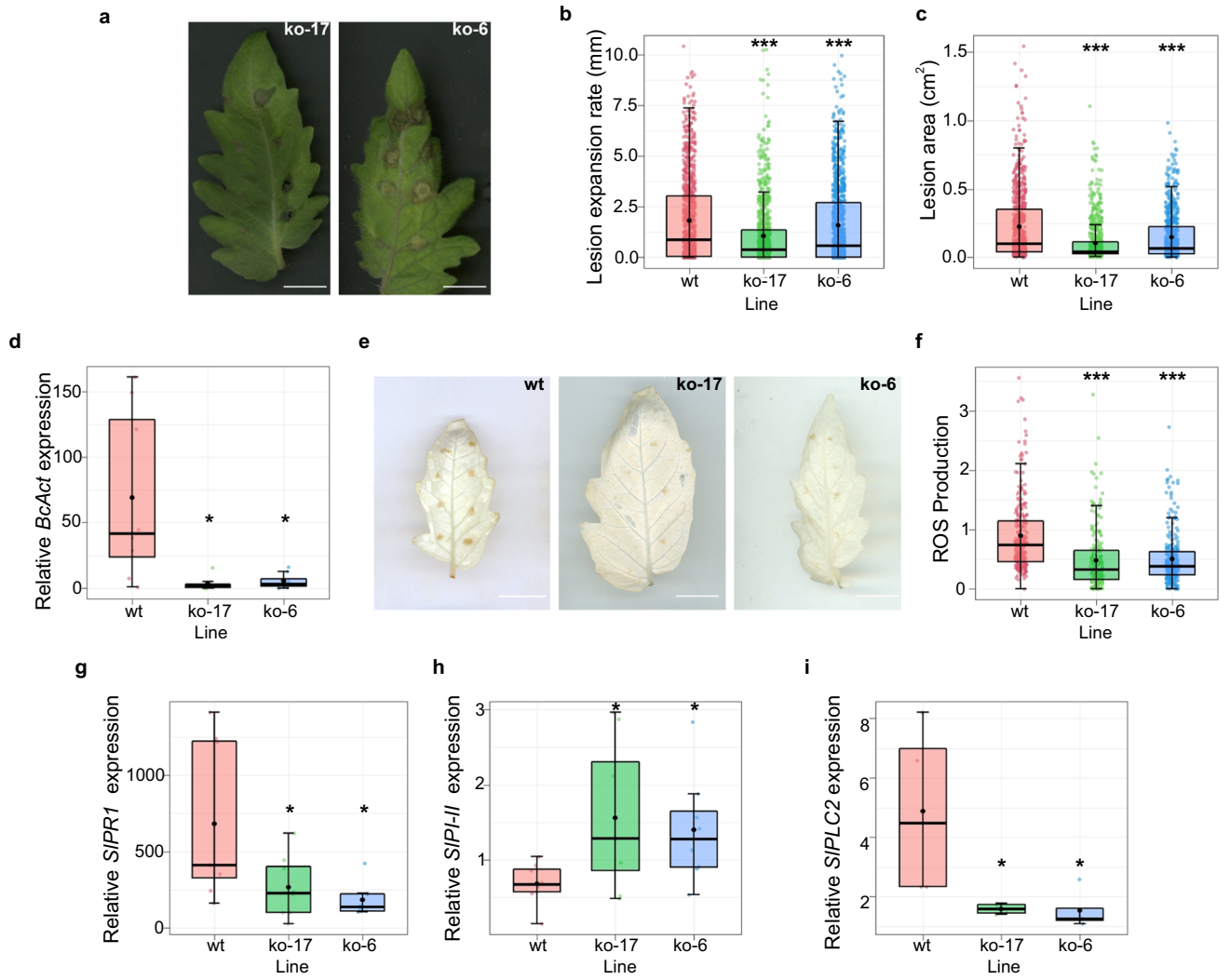

Fig. 2 *SlPLC2 ko* lines show increased resistance to *Botrytis cinerea*. Leaflets of tomato lines ko-17 and ko-6 were infected with droplets of 1×10^6 *B. cinerea* conidia. **a** A representative picture at 72 h post-inoculation (hpi); scale bar = 1 cm. **b** Lesion expansion average diameter of 700–800 inoculation sites between 48 and 72 hpi. **c** Total area diameter at 72 hpi. **d** *BcAct* transcript levels at 72 hpi determined by RT-qPCR ($n = 8$) normalized to *SlAct*. **e** ROS production. Representative picture of DAB-stained leaves 24 hpi; scale bar = 1 cm. **f** Quantification of DAB-stained area (mean gray value/mm^2) was performed using ImageJ 1.3 software. **g** Transcript levels in infected leaves of SA-defense gene *SlPR1a* (72 hpi), **h** JA-defense gene *SlPII-I* (24 hpi) and **i** *SlPLC2* (72 hpi) determined by RT-qPCR ($n = 8$) and normalized to *SlAct*. Asterisks denote that means are significantly different from inoculated WT samples according to a Dunnett Test (*$P < 0.05$; ***$P < 0.0001$)

resistant to Botrytis infections since they produced less ROS, with attenuated SA and intensified JA responses. S genes are generally conserved among species and therefore these results may guide the identification of *SlPLC2* orthologs in other species.

Arabidopsis AtPLC2 is the most abundant PLC isoform, constitutively expressed and rapidly phosphorylated following flg22 recognition (Nühse et al. 2007; Pokotylo et al. 2014). *AtPLC2*-silenced plants displayed normal susceptibility to virulent and avirulent *P. syringae* pv. *tomato* (*Pst*) DC3000 strains but were more susceptible to the type III secretion system-deficient bacterial strain DC3000 hrcC$^-$ and to the non-adapted pea powdery mildew *Erysiphe pisi* (D'Ambrosio et al. 2017). We found that tomato knock-out *SlPLC2* plants showed the same susceptibility to this hemi-biotrophic pathogen, *Pst* DC3000, as wild-type tomato. As mentioned earlier, in a phylogeny analysis AtPLC2 groups within the same clade as SlPLC2 and SlPLC3 (Fig. S1). Like AtPLC2 in *Arabidopsis*, SlPLC3 is the most abundant PLC isoform in tomato. Besides its role during plant defense, AtPLC2 is required for female gametogenesis, embryo development and growth (Kanehara et al. 2015; Li et al. 2015; Di Fino et al. 2017). We are currently in process of editing SlPLC3 to obtain knock-out plants to

evaluate SlPLC3 role on plant development, plant defense and stress.

In summary, using CRISPR/Cas9 we generated tomato plants transgene-free loss-of-function *SlPLC2* stable lines, with improved resistance to Botrytis and normal phenotypic development, without enhanced susceptibility to a bacterial pathogen. We believe these tomato lines represent a promising biotechnological tool, so we will further characterize the role of SlPLC2 in response to other biotic and abiotic stresses.

Author contribution statement EAP perform most of the experiments. JMD design and perform the cloning of the sgRNA into de transformation vector. AADP and SC transform tomato and perform the screening of the lines. MAP and HGR perform *Pseudomonas* infection assays. IC participate in the *Botrytis cinerea* growth and infections. LR assist in the PCR analysis. OM and FV perform the phylogenetic tree. AML design, guided the project and wrote the MS. All the authors read, corrected and approved the manuscript.

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Data availability The data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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Authors and Affiliations

Enzo A. Perk[1] · Andrés Arruebarrena Di Palma[1,3] · Silvana Colman[1,4] · Oriana Mariani[1,5] · Ignacio Cerrudo[1] · Juan Martín D'Ambrosio[1,6] · Luciana Robuschi[1] · Marina A. Pombo[2] · Hernán G. Rosli[2] · Fernando Villareal[1] · Ana M. Laxalt[1]

✉ Ana M. Laxalt
amlaxalt@mdp.edu.ar

Enzo A. Perk
enzoperk@hotmail.com

Andrés Arruebarrena Di Palma
andres_arruebarrena@yahoo.com.ar

Silvana Colman
colmansilvana@gmail.com

Oriana Mariani
ori.mar93@gmail.com

Ignacio Cerrudo
icerrudo@agro.uba.ar

Juan Martín D'Ambrosio
jmda@plen.ku.dk

Luciana Robuschi
robuschi.luciana@gmail.com

Marina A. Pombo
mpombo@agro.unlp.edu.ar

Hernán G. Rosli
hrosli@agro.unlp.edu.ar

Fernando Villareal
fermza@gmail.com

[1] Instituto de Investigaciones Biológicas, CONICET—Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

[2] Instituto de Fisiología Vegetal, INFIVE, Universidad Nacional de La Plata, CONICET, La Plata, Buenos Aires, Argentina

[3] Present Address: Programa de Tecnología, Valorización e Innovación de Productos Pequeros, Instituto de Investigación y Desarrollo Pesquero-CONICET, Mar del Plata, Argentina

[4] Present Address: Laboratorio de Genética, Depto. de Biología, FCEyN, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

[5] Present Address: Plant Nutrition Pflanzenernährung, Martin Luther University, Betty-Heimann-Strasse, 306120 Halle, Germany

[6] Present Address: Section for Plant and Soil Science, Department of Plant and Environmental SciencesCopenhagen Plant Science Center (CPSC)Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark