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Research Article

Transformation of Ponkan Mandarin (*Citrus reticulata* Blanco) by CRISPR/Cas-9-gRNA-CsCS to increase plant resistance to huanglongbing disease

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ABSTRACT

The gene that regulates callose could be modified by CRISPR/Cas9 technology. This research aimed to insert the CRISPR/Cas9-CsCS gene into Ponkan orange genome using Agrobacterium tumefaciens. The explants were soaked in a bacterial suspension for 20 minutes and incubated for 2-3 days. In vitro acceleration growth was conducted with a two-factor completely randomized design. The first factor is the type of explant with three levels (nucellar embryo, zygotic embryo, cotyledon node), and the second factor is the type of media with 6 levels (VMW, MT, MSK0, MSK1, MSK2, MSK3). The results showed of all explant types, the highest plant height average and number of leaves were obtained in a media combination of MS + Kinetin 2 mg L^{-1}, MT, and MS + Kinetin 3 mg L^{-1}. The highest transformation efficiency was in the nucellar embryo explant, while the highest regeneration efficiency was in the zygotic embryo explant. The highest shoot tip grafting percentage was achieved in the cotyledon node explant at 100%. In the grafting phase, the putative transformants before and after artificial bacterial inoculation showed that Ponkan 606 and Ponkan 597 had the highest plant heights, respectively. The intensity of Huanglongbing attacks after bacterial inoculation showed that three genotypes did not show HLB symptoms in the 24th week of observation, namely genotypes 598, 606, and 607. This study concluded that gene transformation in citrus plants produced three genotypes that did not show HLB symptoms.

Keywords: transgenic; *Agrobacterium tumefaciens*; *Citrus Vein Phloem Degeneration* (CVPD)

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INTRODUCTION

Orange is one of the national commodities that has high potential and competitiveness. This is due to the increase in per capita income and awareness of the need for citrus as a source of nutrition. The Indonesian Ministry of Agriculture is currently prioritizing the development of Mandarin orange because of the high imports of Mandarin orange. Ponkan Mandarin (*Citrus reticulata* Blanco) is one of the orange varieties that can be developed and promising because it is one type of Mandarin orange that consumers favor. Ponkan Mandarin has superiority. It has a sweet taste, it is helpful as a source of vitamin C, expectorant, and antitussive because it contains flavonoids such as *hesperidin*

and *narirutin* (Sudo et al., 2021). Ponkan Mandarin has strong growth, these oranges are generally harvested from May to July. Based on data from the BPS (2021), orange production from 2012 to 2021 shows a decrease in production to 192.320 tons, while Mandarin consumption in Indonesia continues to increase. There are several obstacles in increasing the production of Ponkan Mandarin, one of which is the Huanglongbing (HLB) disease. This disease causes a decrease in productivity, quality, and even plant death. Losses can reach up to IDR 120 billion/year and threaten the economy of 65,000 farmers who depend on citrus cultivation (Nurhadi, 2015). Losses due to HLB disease in Florida, United States, are estimated to reach around US$1 billion annually (Li et al., 2020). Bahia experienced even more significant losses of R$1.8 billion (de Oliveira et al., 2013). Huanglongbing in Indonesia was first discovered in 1964. This disease in Indonesia is called Citrus Vein Phloem Degeneration (CVPD). *Candidatus Liberibacter asiaticus* (CLas) is transmitted by an insect vector, i.e., Asian citrus psyllid (ACP, *Diaphorina citri* Kuwayama) (Narouei-Khandan et al., 2016). Plants respond to pathogens by inducing several mechanisms, such as signaling pathway activity and physiological changes, such as cell wall thickening with *callose* accumulation. *Callose* is a crucial defense mechanism against pathogens as it is a common mechanism of cell wall strengthening. Citrus plants with Huanglongbing symptoms infected with CLas will result in heavy *callose* deposition in the vascular tissue (Granato et al., 2019). This can disrupt the development of citrus plants because the process of translocating nutrients to other parts is inhibited (Kosmiatin, 2017). Symptoms of Huanglongbing disease based on the results of research by Ma et al. (2022), namely yellowing shoots with partially yellow/green speckled leaves but some mixed shades of yellow, leaves become short, hardened, and erect, leaf veins become corky, stunted growth, off-season flowering, pale fruit color, thinning of the canopy.

Various methods have been used to control HLB disease. However, these efforts have yet to yield satisfactory results (Ghosh et al., 2023). Citrus plants with resistance genes to Huanglongbing disease have not yet been reported. An alternative to getting citrus plants resistant to Huanglongbing disease is to use a biotechnology approach through genetic engineering techniques by editing the genome. The genome editing process does not result in the introgression of foreign genes in the genome of citrus plants but only silences the expression of specific genes (Kosmiatin, 2017). The CRISPR/Cas9 system is a modern genome editing technology currently being developed, especially in plant breeding. Genome editing in citrus in Indonesia with the CRISPR/Cas9 system has been carried out by Aisyah (2021), which resulted in 55.5% of 20/36 *Japansche Citroen* (JC) transformant plants that did not show any symptoms of HLB at 10 months after artificial inoculation of the pathogen. The working principle of CRISPR/Cas9 for gene editing is sgRNA consisting of tracrRNA that interacts with Cas9 protein, then a complex bond is formed between sgRNA and Cas9 protein containing DNA endonuclease activity. The complicated bond will cause the target dsDNA to be cut off. The served site will be repaired by the *Non-Homologous End Joining* (NHEJ) DNA repair pathway (Horodecka & Düchler, 2021). In this study, CRISPR/Cas9 technology was used to modify the callose synthase gene so that there was no callose accumulation resulting from the activity of *Candidatus Liberibacter asiaticus*, bacterium that causes HLB disease. Therefore, this research aimed to transform the CRISPR/Cas9-CsCS gene into the genome of Ponkan Mandarin plants to increase their resistance to HLB disease with the vector *Agrobacterium tumefaciens.*

MATERIALS AND METHODS

The research was conducted in the Laboratory of Tissue Culture, Molecular Biology, Microbiology and Greenhouse at ICABIOGRAD, Bogor, West of Java Indonesia from June 2021 to March 2023.

Plant material and citrus isolation

Ponkan Mandarin fruits were obtained from the Citrus and Subtropical Fruit Plant Research Center (BALITJESTRO), Kota Batu, East Java. The isolation of nucellar, zygotic

embryos, and nodes cotyledon of citrus was done after the seeds were isolated from fruits aged 11-13 weeks after anthesis with a 2-3 cm fruit diameter. The seed isolation technique used sterilization by burning the fruit in a laminar air flow (LAF), which was done by Kosmiatin (2017). Seed isolation is done by dipping citrus fruits into 96% alcohol and passing it over a bunsen flame five times. Seeds successfully isolated from the fruit were then taken from embryos, separated between zygotic embryos and nucellar embryos, and cultured on VMW media (MS base media modified with the vitamin media formula Morel and Wetmore). The cotyledon node explants were obtained from seed germinated until true leaves appeared, and then the shoot buds were cut and separated from the cotyledon nodes.

Preparation of Agrobacterium tumefaciens vectors for transformation

The *Agrobacterium tumefaciens* vector inserted with the CRISPR/Cas9-gRNA-CsCS gene was prepared by growing bacterial isolates stored as stock on glycerol media. Then, the bacteria were grown on Ludia Bertani (LB) media. Then, *Optical Density* (OD) measurements were taken with OD600=0.2 and OD600=0.02 values (Aisyah, 2021; Wu et al., 2014). The suspension was used to transform nucellar embryos, zygotic embryos, and cotyledon nodes.

Transformation

The transformation was carried out using the method done by Kosmiatin (2017). Nucellar embryos, zygotic embryos, and cotyledon nodes that have been transformed are then co-cultivated for 2-3 days in the dark at room temperature until a halo forms around the explants. Nucellar embryos, zygotic embryos, and cotyledon nodes that have been co-cultivated washed with cefotaxime solution at a concentration of 400 mg L^{-1} and then transferred to Vitamin Morel and Wetmore (VMW) growing media supplemented with cefotaxime antibiotics at a concentration of 400 mg L^{-1}.

Selection of transformant sprouts

Embryos that have been cultured on VMW media with the addition of cefotaxime are then selected on VMW media containing 100 mg L^{-1}hygromycin, and the treatment is carried out for 30 days. The selection of putative transformants was carried out on hygromycin media.

Growth acceleration of transformant sprouts

Growth acceleration of transformant sprouts was carried out after hygromycin selection. In vitro acceleration growth was conducted with a 2-factor completely randomized design. The first factor is the type of explant with 3 levels (nucellar embryo, zygotic embryo, cotyledon node), the second factor is the type of media with 6 levels (VMW, MT, MSK0, MSK1, MSK2, MSK3). VMW (MS base medium modified with Morel and Wetmore media vitamin formula), MT (MS base medium with 10x the vitamin concentration of MS, K (Kinetin).

Shoot tip grafting

Shoot tip grafting is carried out by shoots that have selected hygromycin on growth acceleration media. The grafted shoots were those with at least 3 leaves and were grafted onto 5-month-old JC rootstocks.

Evaluation of resistance of transgenic putative artificial inoculation in vivo

Evaluation of transgenic resistance was carried out in vivo and began with soaking the shoots from 12 weeks after grafting into host-free culture suspension of CLas bacteria that cause huanglongbing disease for 12-16 hours and then grafted by top-working grafting. Observations were made on HLB symptoms on leaves after inoculating and grafting.

Data analysis

The data obtained were analyzed using the F test on T0 transgenic citrus plants, and further tests were done using the Duncan Multiple Range Test (DMRT) at the 5% level. The value of transformation efficiency and regeneration efficiency was calculated using the formula by Putradhika (2019). HLB disease attack intensity was calculated using the formula by Sarwono (1995).

RESULTS AND DISCUSSION

Transformation optimization with several types of citrus explants

Transformation of Ponkan Mandarin plants using the vector *A. tumefaciens* strain EHA 105 with a combination treatment between the type of explant and Optical Density (OD) value. The transformation was carried out by immersing the explants in bacterial suspension for 20 minutes. Optical density is the density of bacteria. The higher the OD value, the higher the number of *A. tumefaciens* contained in the medium (Dewanto & Suhandono, 2016). Optimization in this study was conducted to test the effect of optimal density on the transformation process. According to Guo et al. (2019) concentration of *A. tumefaciens*, duration of infection and co-cultivation are important factors influencing transformation efficiency. Optimization was carried out using different Optical Density values of 0.2 and 0.02. Table 1 shows the results obtained from combining treatments between the types of explants with bacterial density. An OD concentration of 0,02 had higher results, and a suspension with an OD of 0,2 had lower values for all explants and observation parameters. High bacterial cell density can cause uncontrolled *A. tumefaciens* growth, inhibiting explant survival and further reducing transformation efficiency (Asande et al., 2020). Apart from that, the low results obtained were also caused by the size of the explants being too small, so the explants experienced necrosis, which is one of the factors in reducing the level of transformation efficiency (Hartati et al., 2018).

Table 1. CRISPR/Cas9-gRNA-CsCS gene transformation with *A. tumefaciens* vector.

Type of explant	Optical density	Number of explants	%Live explants post-transformation	%Live explants after hygromycin selection	%Germinated explants after transformation
Nucellar	0.20	357	63.03	50.42	5.60
Embryos	0.02	378	72.75	63.23	7.14
Zygotic	0.20	201	85.07	73.13	3.98
Embryos	0.02	226	87.61	79.20	6.19
Cotyledon	0.20	232	100.00	98.28	0.89
Node	0.02	200	100.00	100.00	2.00

Accelerated growth of Ponkan Mandarin plant explants after in vitro transformation

The success of tissue culture is determined by the composition of the media and the addition of plant regulators. According to Handayani et al. (2020) the addition of ZPT in the media is one of the factors that greatly influences the success of network culture. Adding plant growth regulators such as cytokinin in specific amounts and comparisons affects the growth of explants in tissue culture. The use of cytokinin is necessary to stimulate plant shoot multiplication (Lestari, 2011). Analysis of variance showed that the treatment of explant type and media type did not significantly affect the parameters observed, and there was no interaction between explant type and media type.

The growth of explants after transformation was carried out on VMW media, and MS media combined with kinetin at a concentration of 0,1,2,3, mg L^{-1}. Table 2 shows that the treatment of media composition with the type of explants did not significantly affect the parameters of plant height and leaf number. The highest average value of plant height in nucellar embryo explants is 0.760 cm, and the highest average leaf number is 0.850 on MS base media combined with kinetin at a concentration of 3 mg L^{-1}. This is similar to the

study of Mahadi (2016), who finds that adding kinetin at a concentration of 3 mg L^{-1} can influence the height of Kasturi orange shoots. Kinetin with a concentration of 3 mg L^{-1} in the type of nucellar embryo explants can accelerate the growth and development of transformant citrus plants. The highest average value of plant height in the type zygotic embryo explants is 0.793 cm, and the highest average leaf number is 0.953, found in MT media. The average plant height and the leaf number on cotyledon node explants were found in the composition of MS base media combined with kinetin at a concentration of 2 mg L^{-1}, 0.866 cm, and 1.100, respectively.

Table 2. Average plant height and leaf number of putative Ponkan Mandarin transformants in vitro at 12 WAP with different explant types and media composition.

Type of explant	Planting media composition	Plant height (cm)	Leaf number
Nucellar embryos	VMW	0.7b	0.8b
	MT	0.70b	0.8ab
	MS+K0	0.7b	0.7b
	MS+K1	0.7b	0.7b
	MS+K2	0.8ab	0.9ab
	MS+K3	0.80ab	0.9ab
Zygotic embryos	VMW	0.8ab	1.0ab
	MT	0.8ab	1.0ab
	MS+K0	0.7b	0.8ab
	MS+K1	0.7b	0.8ab
	MS+K2	0.70b	0.8ab
	MS+K3	0.8ab	1.0ab
Cotyledon node	VMW	0.8ab	0.9ab
	MT	0.8ab	0.9ab
	MS+K0	0.7b	0.7b
	MS+K1	0.8ab	0.8ab
	MS+K2	0.9a	1.1a
	MS+K3	0.7b	0.8b

Note: Numbers in the same column followed by the same letter are not significantly different at the 5% level of Duncan's test, VMW (MS base medium modified with Morel and Wetmore media vitamin formula), MT (MS base medium with 10x the vitamin concentration of MS, K (Kinetin).

Transformation efficiency, regeneration, and shoot tip grafting success

Transformation efficiency is determined by several factors, namely *A. tumefaciens* concentration, infection, and cocultivation duration (Wen et al., 2022). Cocultivation will optimize *A. tumefaciens* to infect and insert genes in the explant tissue. According to Karthik et al. (2018), the optimal cocultivation period to increase transformation efficiency is two to three days in dark conditions with a temperature of 28°C. Washing using 400 mg L^{-1} cefotaxime antibiotic was then performed after cocultivation to free the explants from A. tumefaciens.

The effective concentration of cefotaxime to eliminate bacteria, according to Kumar et al. (2017) by washing technique is 500 mg L^{-1}, while the concentration of cefotaxime antibiotic 250 mg L^{-1} is effective to suppress the growth of *A. tumefaciens* in subculture media. The transformation efficiency, regeneration efficiency, and percentage of STG success are presented in Table 3. Table 3 shows that the highest transformation efficiency was obtained in the type of nucellar embryo explants at 14,29%. The high value of transformation efficiency is not followed by the high value of regeneration efficiency. Due to the difficulty of eliminating *A. tumefaciens*, it needs to be washed with antibiotics continuously, which can inhibit the growth of transformed explants (Dwinianti, 2013). Several things, such as temperature, humidity, suitability of scion, and rootstock growth,

strongly influence the percentage of STG success. The result obtained in the type of nucellar embryo and zygotic embryos did not grow successfully when grafted because the growth of explants in in-vitro culture was not in accordance with the requirements for STG. There are 7 explants from cotyledon nodes that can be carried out by STG with a success percentage of 100%. The role of rootstock is also important in the success of STG because the scion and rootstock influence each other. The rootstock in this study was able to initiate accelerated growth so that it could meet the need for accumulated food reserves. Studies related to the success of STG conducted by Chalise et al. (2013) in the Nepal region reported that mandarin oranges reached 91.75%.

Table 3. Transformation efficiency, regeneration efficiency, and percentage success of shoot tip grafting (STG).

Type of explant	Initial number of explants	Number of green explants	Number of sprout-resistant explant hygromycin	Number of STG explant	Shoot tip grafting successful explant	Transformation efficiency (%)	Regeneration efficiency (%)	*Shoot tip grafting* success (%)
	A	B	C	D	E	B/A*100	C/A*100	E/D*100
Nucellar Embryos	735	105	14	0	0	14.3	1.9	0
Zygotic Embryos	427	44	21	0	0	10.3	4.9	0
Cotyledon Node	432	36	17	7	7	8.3	3.9	100
Total Explants	1594							

Percentage of disease intensity of plants in putative transformant Ponkan Mandarin

The percentage of attacks and the intensity of plants affected by the disease were obtained from observing symptoms of huanglongbing disease in Six putative transformant lines artificially inoculated with bacteria that cause huanglongbing disease and then carried out by STG. According to Lahlali et al. (2022), the presence of pathogen infections can change the productivity, quality, and quantity of plants in absorbing nutrients. HLB symptoms indicate zinc or manganese deficiency in characteristic spots on the leaves, hardened leaves, shrunken and erect leaves, stunted seedling growth, thin crowns, dead twigs, small fruit, and root rot (Ma et al., 2022). Based on the observation of disease symptoms obtained after artificial inoculation, the Ponkan Mandarin lines had huanglongbing symptoms characterized by shrunken leaves and characteristic spots at weeks 20 (Figures 1a and 1b).

Symptoms of huanglongbing disease on Ponkan transformant lines based on observations presented in Table 4, HLB symptoms on Ponkan Mandarin transformant line began at week 20 after inoculation, namely on KP604 and KP605 lines. Three lines, namely KP598, KP606, and KP607, show no HLB symptoms until week 24 after artificial bacterial inoculation. According to (Keshavareddy et al., 2018), gene transformation in plants is successful if it can produce new plants that can grow normally, are fertile, and express the nature of the new DNA that has been inserted.

Scanning electron microscope (SEM) observations of asymptomatic shoots showed no callose formation and no colonizing bacteria, so the gene completion was successful and mutations occurred in the target gene because no callose compounds were formed (Figure 2a). Symptomatic shoots showed the formation of callose in the vascular tissue with the presence of bacterial colonies in the phloem tissue (Figure 2b). Genetic changes caused by transformation can change different responses in each line. Based on this, plants survive and continue to grow, but it needs to be further observed whether growth will develop or will be inhibited. According to (Muslim et al., 2019), HLB disease was positively

identified after five years of age; this aligns with research conducted in Brazil, which found that the age of citrus plants when HLB disease was first detected was five years old. The difference between symptomatic and non-symptomatic Ponkan Mandarin lines with HLB disease is shown in Figures 1c and 1d.

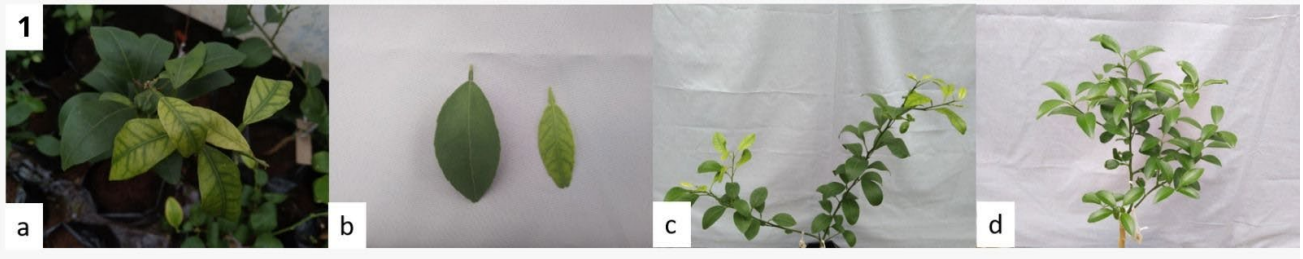

Figure 1. Ponkan Mandarin transformant putative lines at week 20 after inoculation: 1a and 1b HLB symptomatic leaf appearance; 1c. HLB symptomatic plants; 1d. HLB non-symptomatic plants.

Table 4 Percentage of HLB disease intensity of Ponkan Mandarin citrus plants affected by huanglongbing week 24 after inoculation.

Lines	HLB disease intensity (%)	Attack rate
KP597	4.9	Mildly symptomatic
KP598	0.0	Not symptomatic
KP604	12.5	Mildly symptomatic
KP605	9.9	Mildly symptomatic
KP606	0.0	Not symptomatic
KP607	0.0	Not symptomatic

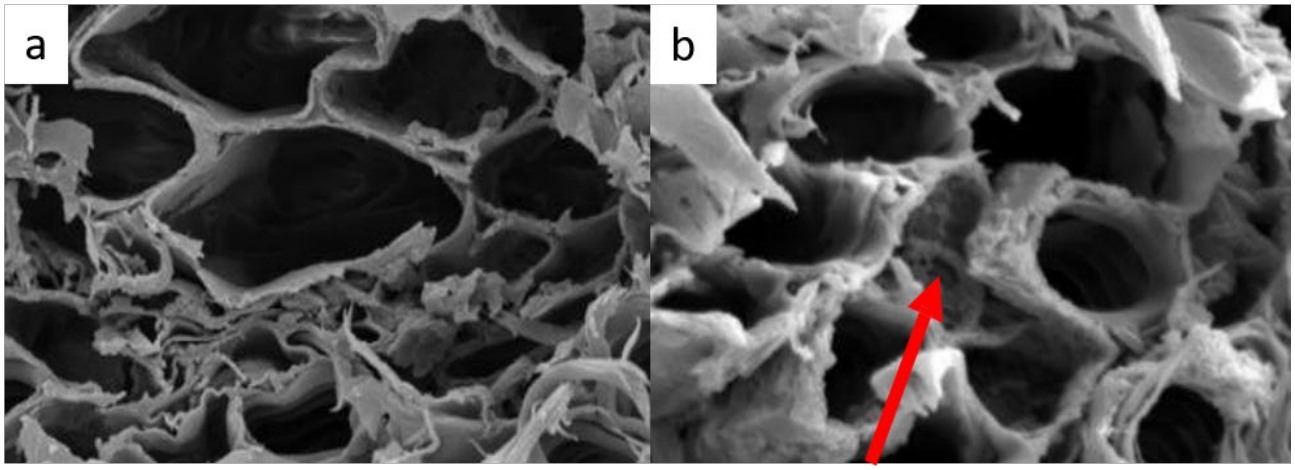

Figure 2. SEM observation of phloem tissue of shoots of Ponkan Mandarin citrus lines inoculated in vivo at 6500x magnification: a. Non-symptoms shoots, b. Symptomatic shoots (red arrow sign).

CONCLUSIONS

CRISPR/Cas9-gRNA-CsCS gene transformation can be performed on several explants (nucellar embryos, zygotic embryos, and cotyledon nodes). The cotyledon node explant type can survive until the shoot tip grafting (STG) and selection after artificial inoculation at the greenhouse. Three lines (KP598, KP606, KP607) were obtained with an attack intensity of 0.00%, meaning they were not symptomatic of HLB disease after artificial bacterial inoculation.

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