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The efficiency of *Agrobacterium*-mediated gene transfer in *Arabidopsis thaliana* mutants

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Original paper

Abstract: A few small molecular weight signals, including jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), regulate the expression of defense-related genes in plants. These signals serve to inhibit the activation of plant defense genes against aggressors and can manipulate the plant's defense signaling pathways. In this study, the impact of acetosyringone on the induction of virulence genes was examined in *Agrobacterium tumefaciens* A348 (MX311) and A348 (MX243) at three different levels: 0, 100, and 200 µM. The concentration that demonstrated the highest induction of virulence genes was then used for transforming *Arabidopsis* mutants using *A. tumefaciens* EHA105, with the aim of inducing virulence genes. Results revealed that *virD2* expression reached its peak at 200 µM acetosyringone, while *virB2* expression was highest at 0 µM. Additionally, transformation experiments indicated that the SA mutants (nahG) exhibited the highest transformation efficiency, while the control plants (Col-0) displayed the lowest efficiency. Therefore, the efficiency of gene transfer in SA-suppression mutants suggests a more significant role for SA in plant defense against pathogens compared to the other hormones. Enhancing gene transfer efficiency in these mutants could unlock the potential for increased expression and production of recombinant proteins compared to the wild type.

Keywords: acetosyringone, in Planta, salicylic acid mutant, jasmonic acid mutant, ethylene mutant, PCR.

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Introduction

Plants display a range of defense mechanisms in response to biotic and abiotic stresses. Some compounds such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play an important role in regulating the defense responses of plants against pathogens, pests and abiotic stresses such as wounds (Loake and Grant, 2007; Balbi and Devoto, 2008) and thus, the expression of genes involved in the defense responses increase with these compounds. SA generally plays an important role in activating plant defense responses against biotrophic and hemibiotrophic pathogens as well as systemic acquired resistance (Grant and Lamb, 2006) while JA and ET are usually associated with plant defense responses to necrotrophic pathogens and herbivorous insects. SA, ET, JA, and camalexin phytoalexin, alone or in together combination, are involved in *Arabidopsis thaliana* defense against various pathogens. SA is also known to protect plants against many pathogens, including fungi, bacteria, and viruses (Kuć, 1982; Tripathi et al., 2019). In several studies, JA concentration has been increased in the pathogen-infected area or damaged tissue and also its external application has been stimulated the expression of genes dependent on plant defense responses (Wasternack, 2007; Siddiqi and Husen, 2019). In addition, plant treatment with ET or its derivatives, as well as ethylene inhibitors, have demonstrated the clear relevance of this plant hormone to plant defense responses(Beckman, 2000; Xu et al., 2018). Although SA and JA/ET defense pathways are antagonistic, evidence of synergistic interactions between these pathways has also been reported (Beckers and Spoel, 2005; Nie et al., 2012). This suggests that the relevance among plant signaling pathways is very complex. On the other hand, in response to *Agrobacterium* infection, the high levels of SA and ET reduce the *Agrobacterium* virulence by inhibiting vir gene expression and T-DNA transfer into plant cells (Yuan et al., 2007; Anand et al., 2008) Therefore, the cross talk of phytohormones plays an important role in the interaction between the host plant and *Agrobacterium*.

The floral dip transformation method, a modified vacuum-infiltration method, was introduced by Clough and Bent (Clough and Bent, 1998) for the transformation of *Arabidopsis*. Immersing the plants containing many unopened flower buds, in the suspension of *Agrobacterium* along with sucrose and Silwet L-77 surfactant, the rate of gene transfer reaches 3-5%. To have a successful gene transfer, it is necessary to pay attention to the growth stage of the plant such as many unopened flower buds, the presence of sugar sources, and the use of surfactants or suction to help penetrate bacteria.

The study of the effect of defense hormones in the presence of *Agrobacterium* leads to understanding the role of those hormones in the efficiency of transformation. Whole plant regeneration from transformed somatic cells occasionally results to generate somatic mutations, so that the presence of phytohormones increases the chances of these mutations occurring (Bent, 2006; Hwang et al., 2017). One solution is to use mutants that lack the ability to produce those hormones. Hence, in this study, we used some *Arabidopsis* mutants to better understand the role of phytohormones, including ethylene, jasmonate, and salicylate, in increasing gene transfer efficiency by *Agrobacterium*. Understanding this will improve our knowledge to increase gene transfer efficiency followed by increase the expression and production of recombinant proteins in plants.

Materials and Methods

Plant materials

jar1 (JA suppression), *etr1-8* (ET-suppression), and *nahG* (SA-suppression) plant mutants and Col-0 (Wild type) were used for transformation. The plants were grown in a growth chamber at 21 °C and a photoperiod of 16/8 darkness/light with a relative humidity of 70%. Jasmonate resistance 1 (JAR1) is a jasmonate-amino synthase that catalyzes the formation of a biologically active jasmonyl-isoleucine (JA-Ile) conjugate (an active form of JA) so that this gene has knocked out in *jar1* mutants (Staswick and Tiryaki, 2004). In *etr1-8* mutants, there is a G-to-A transition in the *ETR1* gene, which results in a stop codon at Trp563 (Hua and Meyerowitz, 1998). On the other hand, nahG plants are containing a gene from the bacterium *Pseudomonas putida* that encodes SA hydroxylase to metabolize SA to catechol, which results in a

dramatic decrease in SA content (Rosas-Díaz et al., 2017).

A. tumefaciens strains and gene construct

Two strains of *A. tumefaciens*, called A348 (MX311) and A348 (MX243), were used. In these strains, pCM110 binary vector carrier Tn3 transposons (containing a gene without lacZ promoter) have been mixed with the promoter of vir genes. Accordingly, A348 (MX243) and A348 (MX311) stains are carrier virB2::lacZ and virD2::lacZ, respectively (Figure 1). These strains were used to determine a concentration of acetosyringone, which induces the expression of vir genes highly, using the measure of β-galactosidase activity assay. Subsequently, those concentrations were used to transform the plant mutants by the *Agrobacterium* EHA105 containing pCAMBIA1105.1.

vir genes induction

A348 (MX243) and A348 (MX311) strains were grown in 5 mL YEP culture medium (10 g/l peptone, 10 g/l yeast extract, 5 g/l NaCl) containing rifampicin (100 µg/ml) and carbenicillin (100 µg/ml) antibiotics at 28 °C overnight with shaking. 0.5 ml of the culture was diluted into 50 ml AB-sucrose minimal medium (50 ml 20X AB-buffer (60 g/l K2HPO4, 20 g/l Na2HPO4, pH=7), 50 ml 20X AB-salts (20 g/l NH4Cl, 6 g/l MgSO4.7H2O, 3 g/l KCl, 0.2 g/l CaCl2, 50 mg/l FeSO4.7H2O), 900 ml 0.05% sucrose solution) containing rifampicin and carbenicillin antibiotics and grown overnight at 28 °C until the bacteria were in late log phase (OD600 = 0.8). The bacteria were centrifuged at 9000 xg for 5 min and the pellet was re-suspended in two volumes of induction medium (1X AB-salts, 2 mM phosphate buffer (pH=5.6), 50 mM 2-(4-morpholino)-ethane sulfonic acid, 0.5% glucose) containing the different concentrations of acetosyringone (0, 100 and 200 µM) and shaken very gently (approx. 50 rpm) for 14-24 h at 25 °C (Gelvin, 2006).

β-galactosidase activity assay

After induction of vir genes, an aliquot of them was centrifuged for 1 min and then, re-suspended in a final volume of 4 mL Z-buffer (16.1 g/l Na2HPO4.H2O, 5.5 g/l NaH2PO4.H2O, 0.74 g/l KCl, 0.246 g/l MgSO4.7H2O, 2.7 ml β-mercaptoethanol, pH=7), and OD600 was adjusted to 0.1-0.25. Two drops of 0.1% SDS and four drops of chloroform were added to the 2 ml cell culture, vortexed and incubated in a 30 °C water bath for 10 min. In the next step, 400 µl of O-Nitrophenyl-β-D-galactoside (ONPG, 4 mg/ml in Z-buffer) solution was added, vortexed, and started timing until 60 min to detect β-galactosidase activity. Finally, the reaction was terminated by the addition of 1 ml 1 M Na2CO3 and read the absorption at both 420 and 550 nm. The β-galactosidase activity was calculated as follows (Miller, 1972):

$$Miller\ unit = \frac{1000\ (A420\ nm - 1.75 \times A550\ nm)}{time\ (min.) \times A600}$$

Plant transformation

The floral dip method was used to plant transformation. First, the vir genes of the *Agrobacterium* EHA105 containing the pCAMBIA1105.1 vector were induced as above in a 200 µM concentration of acetosyringone (as treatment) and 0 µM concentration of acetosyringone (as control). The bacteria were centrifuged at 9000 xg for 5 min, resuspended in 5% sucrose solution containing 0.02% (v/v) silwet L-77.

Figure 1. Structure and organization of Tn*3*-HoHol. The coding region within the element and the transcriptional orientation of each gene are indicated by an arrow. *tnpR* and *bla* are wild-types, while *tnpA* is non-functional owing to the *lac* sequences inserted into its 3' end. The *lac* operon sequences are intact but lack a functional promoter. Translation can potentially initiate at *virB2 / virD2* that occurs upstream of the *lacZ* structural gene. Symbols: IRL, left-inverted repeat; IRR, right-inverted repeat; *lac*ZYA, *E. coli lac* operon; *tnpA*, transposase; *tnpR*, resolvase; *bla*, β-lactamase.

Afterward, the plant mutants were inoculated with bacteria suspension. To maintain 100% moisture, the plants were kept in dark boxes covered with plastic for 24 hours and finally, the plants returned to normal growth conditions. After a week, this work was repeated to increase the efficiency of the transformation.

Screening of putative transgenic plants

Hygromycin resistance analysis

Surface sterilization of harvested seeds was done with 70% ethanol for 1 minute and afterward with 5% sodium hypochlorite for 10 minutes, then washed several times with sterilized water. The sterile seeds were cultured in an MS plant tissue culture medium containing hygromycin antibiotic (50 µg/mL) at 4 °C for two days and then, were transferred to a growth chamber at 20-22 °C with photoperiod 8/16 darkness/light. After 10 days, grown seedlings and green were transferred to the pot.

PCR analysis

The DNA extraction from the putative transgenic plant mutants was carried out using the Dena Zist kit (S-1030-1) according to the manufacturer's instructions. To confirm the presence of hyg in the plant mutants, a PCR reaction was performed using specific primers. To confirm the absence of bacterial contamination of the plants, spect specific primers were used (Table 1). Finally, the PCR products were run on the 1% agarose gel.

Statistical analysis

All experiments were carried out in a completely randomized design with two biological replications and three technical replications. Analysis of variance of the data obtained from the experiment and comparison of the mean of treatments with Tukey test at P-value ≤ 0.01 were performed using SAS 9.0 software and the charts were drawn using GraphPad prism software.

Results

The induction of vir promoter activity in the MX311 and MX243 strains

In the MX311 strain, the results showed that the highest and lowest of β-galactosidase activity was at 200 and 0 µM concentrations of acetosyringone respectively, while in the MX243 strain, the highest and lowest of β-galactosidase activity was at 0 and 100 µM concentrations of acetosyringone respectively (Figure 2).

Also, the results of the analysis of variance showed that β-galactosidase activity in the MX311 strain has a significant difference in the different concentrations of acetosyringone at P-value ≤ 0.01 (Table 2). However, β-galactosidase activity in MX243 strain had a significant difference among 0 and 100 µM concentrations of acetosyringone at P-value ≤ 0.01, while no significant difference was observed between each of concentrations of 0 and 100 µM with the concentration of 200 µM at P-value ≤ 0.01 (Figure 2 and Table 3).

Table 1. The sequences of specific primers used in PCR.

Gene	Sequence	Tm (°C)	PCR product (bp)
hyg	F: 5′-GATGTTGGCGACCTCGTATT-3′ R: 5′-GTGCTTGACATTGGGGAGTT-3′	63.7 63.9	450
spect	F: 5′-ATTTGCCGACTACCTTGGTG-3′ R: 5′-GAACATAGCGTTGCCTTGGT-3′	63.7 63.9	450

Table 2. Analysis of the variance of β-galactosidase activity in different concentrations of acetosyringone in MX311 strain.

Source of changes	DF	Sum of Squares	Mean Square	*P-value*
Treatment	2	2230874.918	1115437.459	< 0.0001
Error	6	38.987	6.498	

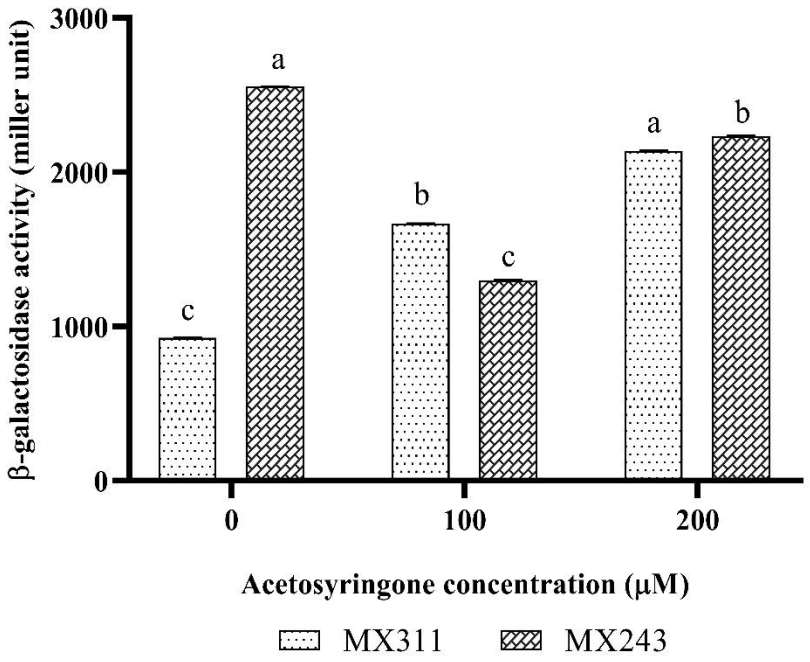

Figure 2. β-galactosidase activity in the presence of different concentrations of acetosyringone in MX311 and MX243 strains. Letters show a significant level at *P-value* of ≤ 0.01.

Table 3. Analysis of the variance of β-galactosidase activity in different concentrations of acetosyringone in MX243 strain.

Source of changes	DF	Sum of Squares	Mean Square	*P-value*
Treatment	2	2550991.162	1275495.581	< 0.0001
Error	6	38.599	6.433	

Molecular analysis and resistance to hygromycin antibiotic

Resistance test to hygromycin antibiotic

By counting the number of putative transgenic plants on the culture medium containing hygromycin, the transformation efficiency was calculated according to Lin et al. (Lin et al., 2009) method. The results showed that the plant mutants, which were inhibited in SA biosynthesis (nahG), had the highest transformation efficiency among other mutants, and the lowest transformation efficiency was related to the control ecotype (Col-0) in the absence of acetosyringone (Figure 3). The results of mean comparisons and variance analysis showed that there is a significant difference among plant mutants in the presence of acetosyringone at P-value ≤ 0.01, while there isn't a significant difference among the plant mutants in the absence of acetosyringone at P-value ≤ 0.01.

Molecular analysis by PCR

To confirmation of putative transgenic plants, the extracted DNA from each plant mutant was used as the template of PCR reaction using the specific primers of hyg. Transgenic plants were confirmed with the presence of a 450 bp fragment (Figure 4). The non-transgenic plant was a negative control for hyg. Also, the absence of *Agrobacterium* contamination was confirmed using the extracted DNA from transgenic plants as the template of PCR and the specific primers of spect. The absence of 450 bp fragment was confirmed the absence of *Agrobacterium* contamination (data not shown).

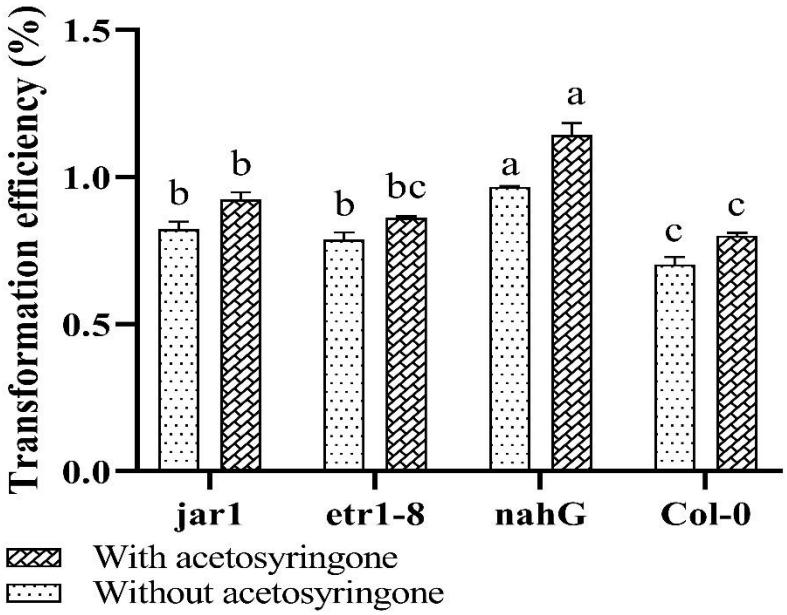

Figure 3. Comparison of transformation efficiency in mutant plants using hygromycin antibiotic resistance test. Letters show a significant level at *P-value* of ≤ 0.01.

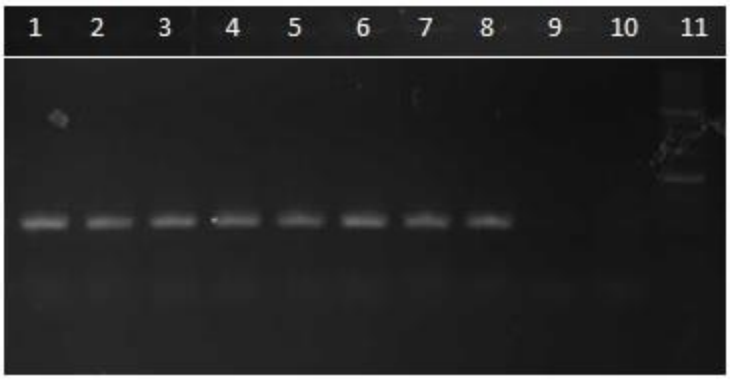

Figure 4. Confirmation of putative transgenic plants using specific primers of *hyg*. Lane 1-4: DNA from transgenic plants of Col-0, *etr1-8*, *nahG*, and *jar1* in the present of acetosyringone, respectively. Lane 5-8: DNA from transgenic plants of Col-0, *etr1-8*, *nahG*, and *jar1* in the absence of acetosyringone, respectively. Lane 9: DNA from the non-transgenic plant. Lane 10: negative control in the absence of DNA. Lane 11: 1 kb molecular ladder.

4. Discussion

The vir expression induction with phenolic compounds, such as acetosyringone, naturally is a prerequisite for transformation, and in normal conditions, a minimal medium is used for the induction of vir gene (Gelvin, 2006). Generally, the optimum temperature for the induction of vir (25 °C) is lower than the optimum temperature for *Agrobacterium* growth (28-30 °C), in which this principle was followed in this experiment. Also, increasing the expression of the *virD2* results in the transfer of the T-DNA more efficiently to the host cell and thus increases the efficiency of the transformation, which according to the results of this study, increases the concentration of acetosyringone enhanced the induction of *virD2*. On the other hand, *Vir B1-11* genes are involved in forming a communication channel between the bacterial cell and the plant cell to transmit T-DNA, resulting in the minimal expression of Vir B2 would

be sufficient for this operation. Also, the sugars like glucose in the presence of limited concentrations of acetosyringone increase the induction of vir, which mainly is used instead of sucrose in a plant growth medium (Wang, 2006).

Identification of the main hormones involved in plant defense provides an ideal model for dealing with interactions between pathogenic bacteria and plants. Plant hormones of SA, JA, and ET are essential for contributing to regulating plants' defense(Glazebrook, 2001; Thaler et al., 2004). After infection, the amount of SA increases in response to acquired systemic resistance, which results in prolonged resistance to the pathogen (Durrant and Dong, 2004). Thus, in this study, disrupting the pathway of SA biosynthesis in the mutants of *nahG*, increased their sensitivity against Agrobacterium and the transformation efficiency. In *nahG* plants, the expression of PR-genes has greatly reduced and they thus exhibit enhanced susceptibility to different pathogens (Heck et al., 2003; Dobon et al., 2013). In addition, it has been reported that the accumulation of ET decreases after infection with *Xanthomonas* compestris at *nahG* plants (O'Donnell et al., 2003). Therefore, it shows that *nahG* plants display hormonal disorders related to SA and ET, two important hormones in plant defense, and it may be a reason to increase the transformation efficiency of these plants compared to others in this study. In a study, *Agrobacterium*-mediated transformation efficiency is increased in *sid2* and *nahG* plants, which both are deficient in salicylic acid production (Rosas-Díaz et al., 2017). Interestingly, lack of salicylic acid production is thought to not affect bacterial growth, bacterial attachment to plant cells, inhibiting the expression of vir genes, and virulence (Hwang et al., 2017; Rosas-Díaz et al., 2017).

The role of JA response in resistance to some pathogens in several plants such as *Arabidopsis* (Thomma et al., 1998), tomatoes (Diaz et al., 2002) Norway spruce (Kozlowski et al., 1999) and barley (Mitchell and Walters, 1995) is reported. Mechanisms that affect the response of JA can induce pathogenesis-related genes in *Arabidopsis* (Thomma et al., 1998; Hamamouch et al., 2011). Therefore, JA has a lower role in plant defense against pathogens than SA, as they are mutually antagonistic(Li et al., 2019). Therefore, our results showed the transformation efficiency of JA-suppression mutants is lower than SA-suppression mutants. In addition, although SA and JA show a negative regulation of each other, they also sometimes have synergistic effects (Dobon et al., 2013).

ET controls the extent and development of plant disease symptoms after inoculation with pathogenic bacteria or fungi (Bent et al., 1992; Zhou et al., 2019). Therefore, it can be assumed that the *etr1-8* mutants should have high efficiency in transformation with *Agrobacterium* compared to the control. Further, studies indicate that the growth of *Agrobacterium* was not affected by ET, but the presence of ET at the start of the infection with *Agrobacterium* showed significant inhibitory activity in the vir expression (Nonaka et al., 2008). Such inhibitory effects can be eliminated through supplementation with acetosyringone, as a vir inducer (Nonaka et al., 2008). These observations indicate that ET affects the interaction between *Agrobacterium* and plants due to its inhibitory effects on bacterial pathogenicity. Therefore, in plant defense against pathogens, SA plays a more important role than JA and ET (Anand et al., 2008). As expected, in the SA mutants, the efficiency of transformation is higher than other mutants due to the lack of SA production.

Up to today, the low production yield of recombinant protein in plants has become a challenge and this encouraged us to look up other aspects of increasing gene transfer efficiency to increase the expression and yield of recombinant proteins. In this study, one of these aspects was found by determining the role of defense hormones in the efficiency of *Agrobacterium*-mediated gene transfer. According to the results, when in the absence of salicylate, the efficiency of gene transfer increases, these results can be very promising for the production of a recombinant protein. Hence, by increasing the efficiency of gene transfer, the amount of recombinant protein produced may increase (Zhao et al., 2017).

Conclusion

In this study, the effect of acetosyringone on increasing the transformation efficiency, three old-day culture was investigated using YEP, AB-sucrose, and induction media based as described by

Gelvin (Gelvin, 2006).The results showed that the presence of acetosyringone increased the efficiency of the transformation. Also, given the role of plant hormones of SA, JA and ET in plant defense against pathogens and disrupting their pathway of biosynthesis causes plant sensitivity to the pathogen. According to the findings of this study, in mutants with impaired regulation of biosynthesis of each of the above hormones exhibited higher transformation efficiency than the control plants. The use of mutants offers an effective approach to enhance the efficiency of *Agrobacterium*-mediated gene transfer because direct application of the hormone may activate some other defense mechanisms potentially impede gene transformation s by *Agrobacterium*. On the other hand, the presence of inducer compounds like acetosyringone has been shown to significantly increase the efficiency of the transformation when compared conditions lacking these inducers. Finally, since SA-suppression mutants have the highest transformation efficiency compared to others, it can be concluded that SA may play a greater role in plant defense against *Agrobacterium* than two other hormones. Therefore, the use of mutants lacking the ability to biosynthesize SA is a promising strategyto increase the production of recombinant proteins in the plant.

Supplementary Materials:

No supplementary material is available for this article.

Author Contributions:

Conceptualization, M.S.T. and M.M.S.; methodology, M.S.T.; software, M.S.T. and M.M.S.; validation, M.S.T. and R.S.; formal analysis, M.S.T. and R.S.; investigation, M.M.S and R.S.; resources, M.M.S.; data curation, M.S.T., M.M.S. and R.S.; writing—original draft preparation, M.S.T.; writing—review and editing, M.S.T., M.M.S. and R.S.; visualization, M.S.T., M.M.S. and R.S.; supervision, M.M.S. and R.S..; project administration, M.M.S.; funding acquisition, M.M.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

There is no conflicts of interest.

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کارایی انتقال ژن به‌واسطه آگروباکتریوم در موتانت‌های آرابیدوپسیس تالیانا

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چکیده: تعداد کمی از سیگنال‌های با وزن مولکولی پایین، از جمله جاسمونیک اسید (JA)، سالیسیلیک اسید (SA) و اتیلن (ET)، بیان ژن‌های مرتبط با دفاع را در گیاهان تنظیم می‌کنند. این سیگنال‌ها جهت مهار فعالسازی ژن‌های دفاعی گیاه در برابر مهاجم‌ها فعالیت می‌کنند و می‌توانند مسیرهای سیگنالینگ دفاعی گیاه را دستکاری کنند. در این مطالعه، تاثیر استوسیرینگون بر روی القا ژن‌های بیماری‌زا در آگروباکتریوم تومه‌فاشینس سویه‌های A348 (MX311) و A348 (MX243) در سه سطح مختلف ۰، ۱۰۰ و ۲۰۰ میکرومولار مورد ارزیابی قرار گرفت. غلظتی که بالاترین القا ژن‌های بیماری‌زا را داشت متعاقبا برای القا ژن‌های بیماری‌زا آگروباکتریوم تومه‌فاشینس EHA105 جهت ترانسفورماسیون موتانت‌های آرابیدوپسیس استفاده شد. نتایج نشان داد که بیان virD2 در غلظت ۲۰۰ میکرومولار استوسیرینگون به حداکثر مقدار خود رسید در حالی که بیشترین بیان virB2 در غلظت ۰ میکرومولار بود. علاوه بر این، آزمایشات ترانسفورماسیون نشان داد که موتانت‌های سالیسیلیک اسید (nahG) بالاترین کارایی ترانسفورماسیون را نشان دادند در حالی که گیاهان کنترل (Col-0) پایین‌ترین کارایی را داشتند. بنابراین، کارایی انتقال ژن در موتانت‌های سرکوب کننده SA در مقایسه با سایر هورمون‌ها نقش قابل توجه‌تر SA در دفاع گیاه در برابر پاتوژن‌ها را پیشنهاد می‌دهد. افزایش کارایی انتقال ژن در این موتانت‌ها می‌تواند راهی برای افزایش بیان و تولید پروتئین‌های نوترکیب در مقایسه با نوع وحشی باز نماید.

کلمات کلیدی: استوسیرینگون، In planta، جهش‌یافته اسید سالیسیلیک، جهش‌یافته اسید جاسمونیک، جهش‌یافته اتیلن، PCR.

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