Effects of Plant Growth Regulators On Transient Expression of Foreign Genes In Nicotiana Benthamiana L. Leaves

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Short report

Keywords: Plant growth regulators, Agrobacterium-mediated transformation, Geminivirus-derived vector, Transient expression

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Effects of plant growth regulators on transient expression of foreign genes in *Nicotiana benthamiana L.* leaves

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Abstract

Background: In the last decades, autonomously replicating expression vectors based on plant geminivirus has been widely used as one of the most effective expression vectors in plant transient expression**.** Compared with those non-replicating expression vectors, autonomously replicating expression vectors based on plant geminivirus have many advantages. By using the bean yellow dwarf virus-derived expression vector and green fluorescent protein as a reporter, we investigated the effects of α-naphthalene acetic acid, gibberellins, and 6-benzyladenine, as three common plant growth regulators, on the efficiency of plant transient expression and biomass during the process of transient expression in *Nicotiana benthamiana L.* leaves.

Results: With the increase of α-naphthalene acetic acid, gibberellins, and 6-benzyladenine (from 0.1 to 1.6 mg/L), the fresh weight, dry weight, and leaf area of the seedlings increased first and then returned to the levels similar to the controls (without plant growth regulators treatment). α-naphthalene acetic acid at 0.2 and 0.4 mg/L can enhance the level of transient expression of green fluorescent

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protein, which peaked at 0.4 mg/L α-naphthalene acetic acid and was increased about 19%, compared to the controls. Gibberellins at 0.1 to 0.4 mg/L can enhance the level of transient expression of green fluorescent protein, which peaked at 0.2 mg/L gibberellins and was increased about 25%. However, the 6-benzyladenine application led to decrease in the level of transient expression of green fluorescent protein.

Conclusions: The moderate concentration of appropriate plant growth regulators could be beneficial to the expression of foreign proteins from the *Agrobacterium*-mediated transient expression system in plants. Thus, appropriate plant growth regulators could be considered as an exogenous component to be applied in recombinant protein production by plant-based transient expression systems.

Keywords: Plant growth regulators, *Agrobacterium*-mediated transformation, Geminivirus-derived vector, Transient expression

Introduction

Transient expression technology refers to the technology of introducing the target gene into recipient cells to establish a temporary high-efficiency expression system so that the target gene can be expressed in a relatively short time. When using transient technology to express the target gene, the transformed target gene is not integrated into the genome, which saves the time of genetic transformation and screening (Chen et al. 2013).

Production of the desired protein via transient expression has obvious advantages, including less

time with more protein expression, consistency in protein accumulation with lower cost, and easy manipulation without any biosafety concerns, compared to develop stable transgenic lines with time-consuming procedures (Xia et al. 2020). And, in recent years, plants have been emerged as an alternative platform for the production of recombinant proteins to meet the worldwide demand for protein-based pharmaceuticals (Chen 2008). Compared to mammals, yeast, and bacteria cells, the platforms for plant-based recombinant protein production by transient expression is more reliable, scalable, low-cost, and safe (Damaj et al. 2020). From the current reports, the transiently expressed recombinant protein in plants can be extracted within 1-2 weeks after transformation (Leuzinger et al. 2013). Especially today, when new pathogens and diseases are prone to sudden outbreaks, the production of medicinal proteins or antibodies in plants by transient expression system is of great significance for social security and disease treatment (Peyret and Lomonossoff 2015).

In the last decades, autonomously replicating expression vectors based on plant geminivirus has been widely used as one of the most effective expression vectors in plant transient expression (Hefferon 2014; Rybicki and Martin 2014). Compared with those non-replicating expression vectors, autonomously replicating expression vectors based on plant geminivirus can produce a large number of copies of target genes after transformation, thus largely enhancing the expression level of the desired protein (Abrahamian et al. 2020). For example, the bean yellow dwarf virus (BeYDV) is one of the geminiviruses in the genus Megaviruses. Its genome is circular single-stranded DNA (ssDNA) with a

size of about 2561 bp. After infecting the host plant, BeYDV quickly replicates to a high copy number in the host cell nucleus by a rolling circle replication mechanism (Chen et al. 2011; Zaidi and Mansoor 2017). Based on the above characteristics, BeYDV-derived expression vectors are constructed, which contain the replication-related elements of BeYDV and an expression cassette for a protein of interest (Zhang and Mason 2006; Baltes et al. 2014). Many works have shown that using the BeYDV-derived expression vectors in plant transient expression can generate a large number of target gene copies in plant host cells, thus greatly improving the efficiency of plant transient expression (Richter et al. 2016; Hanley-Bowdoin et al. 2013).

Besides the development or modification of new expression vectors, some efforts are also attempted to further improve the efficiency of transient gene expression in plants. These include the optimization of internal or external factors that could affect the efficiency of plant transient expressions, such as the concentration of *Agrobacterium tumefaciens*, temperature, light intensity, and humidity (Maleki et al. 2018; Fujiuchi et al. 2016). Although some of these internal factors can be easily controlled, it is still difficult to obtain a higher protein yield from transient expression in plants by modifying external environmental factors, especially when the plants used for transient expression were grown in the field. Thus, developing some novel methods that can be easily applied is still needed to enhance the utility of plant-based transient expression systems as recombinant protein factories.

Plant growth regulators (PGRs) are a kind of chemical substance synthesized artificially, which

72 can regulate the growth and development of plants by exogenous application (Gong et al. 2021). Three
73 kinds of PGRs are commonly used in agriculture for enhancing the production of crops. α-naphthalene
74 acetic acid (NAA), which belongs to the synthetic branch of auxins, is a naphthalene derivative widely
75 used to stimulate plant growth, prevent the premature fall of fruits, and increase the yield of crops
76 (Guan et al. 2011). Gibberellins (GA_3) has long been known to promote the growth and development
77 of plants, which has been applied to regulate seed germination, organ elongation, flowering, and fruit
78 maturity by affecting mitotic frequency or cell enlargement (Hedden and Sponsel 2015). 6-
79 Benzyladenine (6-BA) is widely involved in mediating various physiological processes of plants, such
80 as plant stem and root growth, cell proliferation, chloroplast development, and biomass distribution
81 (Werner et al. 2010; Hwang et al. 2012), thus severing as the regulator for promoting plant growth,
82 increasing chlorophyll content, and delaying senescence (To and Kieber 2008). More importantly,
83 these PGRs are lower cost, relatively cheap, and are easy to apply in the field. However, information
84 about the effects of PGRs on the efficiency of plant transient expression is very limited.

85 Therefore, in this study, by using the BeYDV-derived expression vector and green fluorescent
86 protein (GFP) as a reporter, we investigated the effects of NAA, GA_3, and 6-BA, as three common
87 PGRs, on the efficiency of plant transient expression and biomass during the process of transient
88 expression. We believe that this study would help provide a reference for how to utilize PGRs to
89 improve the yields of recombinant protein from plant transient expression.

Materials and methods

Experimental material

Cultivation of plant materials

Seeds of *Nicotiana benthamiana L*. were collected and preserved in the laboratory. Two or three seeds of *Nicotiana benthamiana* were planted into the peat pell in the seedling box and were cultured in a culture room with a temperature of 25℃, a humidity of 50 %, and a day and night period of 16/8 h. Hoagland nutrient solution was applied. The 3-week-old seedlings were moved from the seedling box to provide more sufficient space for further growth until they were ready to be treated at the 4th week of growth.

The geminivirus-derived expression vector and *Agrobacterium* transformation

The expression vector based on BeYDV was presented by Professor Mason of Arizona state university. GFP gene was constructed downstream of the 35S promoter in the vector as the reporter gene. The engineering strain of *Agrobacterium* used in the present works was LBA4404. The expression vector based on BeYDV was transformed into this *Agrobacterium*, and the antibiotic resistance gene of the vector was used for screening and subculture.

Preparation of bacterial cultures

Agrobacterium tumefaciens LBA4404 strains harboring the BeYDV-derived expression vector with the GFP gene were streaked on *Agrobacterium* rhizogene medium (YEB) Agar plates containing

kanamycin (50 ug/ml), rifampicin (25 ug/ml), and chloramphenicol (25 ug/ml). After 24 h growth, the monoclone was picked out from the YEB agar plate and inoculated into 50 mL YEB broth liquid medium with the antibiotics at 28°C overnight in a shaker at 180 rpm. The bacteria were collected by centrifugation at 5000 rpm for 10 min and then washed by infiltration buffer (10 mM MES-KOH, pH 5.5; 10 mM MgSO4; 100 uM Acetosyringone). After then, the bacteria were collected again and re-suspended in the infiltration buffer. The bacterial concentrations were determined by measuring optical density (OD) at 600 nm and were diluted to 0.3 with the infiltration buffer.

PGRs treatment and plant transformation

The types of plant growth regulators used in this experiment included NAA, GA_3, and 6-BA. The spraying concentration of each growth regulator is 0 mg/L, 0.1 mg/L, 0.2 mg/L, 0.4 mg/L, 0.8 mg/L and 1.6 mg/L, respectively.

Three plant growth regulators, NAA, GA_3, or 6-BA, with different concentrations, were prepared with deionized water and were sprayed on the leaves of 4-week-old seedlings with uniform growth. In the control group, the same amount of distilled water was sprayed, and isolation barriers were set in each spraying to avoid cross-influence. Spray application was performed until the leaf was wet and the solution ran off. The seedlings were sprayed three times continuously daily and the next spray application was performed after 72 hours. The growth of plants sprayed was observed, and the parameters of fresh weight, dry weight, and leaf area were measured.

At 9 d after the first spray application with PGRs, a small gap was slightly cut in the lower epidermis along the main vein of the leaf, which is 1/3 away from the leaf base and 0.5 cm away from the main vein. Two mL *Agrobacterium* suspension was slowly injected into the leaf through the gap of the lower epidermis with a sterile syringe without a needle. After infiltration, the plants were moved back to the culture room, and the expression of GFP was monitored at 4th after infiltration.

Determination method

Determination of biomass

An analytical balance was used to measure the fresh weight of the aerial part of tobacco seedlings. For the determination of dry weight, the aerial part of seedlings was put in an oven for deactivation of enzymes at 100℃ for 20 minutes, dried to constant weight at 70℃, and then taken out for weighing the dry weight. For the determination of leaf area, photos of all leaves on each seedling were taken by camera, and Photoshop software was used to measure the leaf area.

Detection of GFP expression

At the 4th after the injection of the infiltration buffer, the lower surface of the infiltrated leaf was placed under a Leica fluorescent stereomicroscope (Leica Microsystems Ltd. DFC450 C). A region of 1 cm away from the original injection site was selected. This region was excited at a wavelength of 450-490 nm and the emission spectrum between 500-550 nm was recorded.

Data analysis

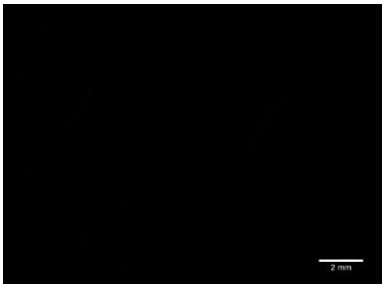
The leaf area was measured by Photoshop CS5 software, the GFP fluorescence was analyzed by Image J software, and the data were analyzed by One-way ANOVA with IBM SPSS Statistics 19 software. LSD and Duncan methods were used for multiple comparisons and significance analysis, and the significant difference was expressed by $P < 0.05$. The value obtained is the average value of at least 3 independent experiments, and the data are expressed as mean value standard error (SE).

Results

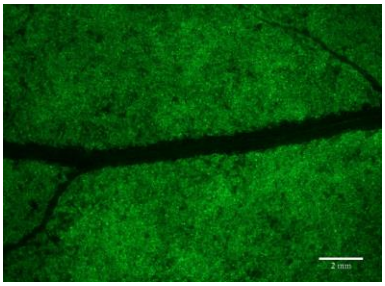
Effect of NAA on transient expression and growth of *Nicotiana benthamiana L.* seedlings

GFP expression in the leaves of *Nicotiana benthamiana L*. seedlings by the BeYDV-derived expression vector.

In this study, strong GFP expression was observed in the leaves of *Nicotiana benthamiana L*. that were infected with LBA4404 carrying GFP gene in the BeYDV-derived expression vector, while green fluorescence was not observed in the leaves infiltrated by the *Agrobacterium* LBA4404 strain without expression vector (fig.1). Thus, the observed green fluorescence was specifically from the expression of the GFP gene.



LBA4404 strains without BeYDV-derived expression



LBA4404 strains containing BeYDV-derived

Fig. 1 The fluorescent expression of GFP in LBA4404 strains without BeYDV-derived expression vector (left) and with LBA4404 strains containing BeYDV expression vector (right) Bar = 2 mm, same below

Effect of NAA on the growth of tobacco seedlings

It can be seen from fig.2 that treatment with 0.1 mg/L NAA did not significantly affect the biomass of the seedlings, compared to the control (treatment with water). NAA at 0.2 or 0.4 mg/L significantly increased the fresh weight, dry weight, and leaf area of tobacco seedlings, and the biomass of the seedlings treated with 0.4 mg/L NAA was significantly higher than that of the seedlings treated with 0.2 mg/L NAA. However, the 0.8 mg/L NAA failed to increase the fresh weight, dry weight, and leaf area of the seedlings. When the concentration of NAA reached 1.6 mg/L, the biomass of the seedlings presented a decrease, compared with the control. The fresh weight and dry weight of the seedlings treated with 1.6 mg/L NAA was decreased significantly by 29.12% and 23.54%, respectively, while the leaf area was decreased significantly by 18.56%, compared with the control.

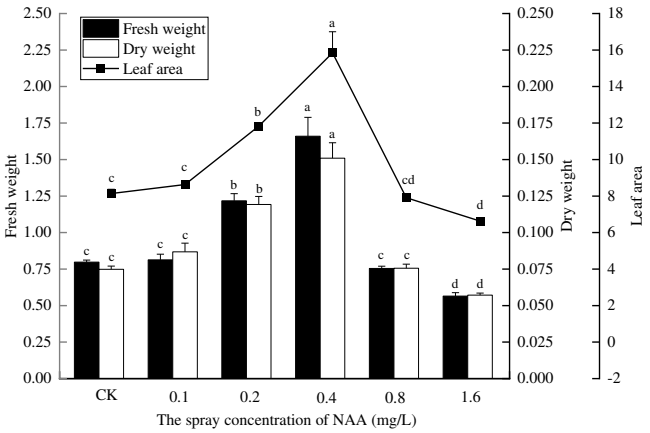


Fig. 2 Changes of fresh weight, dry weight, and leaf area of the seedlings under different concentrations of NAA

Different lowercase letters indicated that there are significant differences in the same parameter among the treatment with different concentrations at the $P < 0.05$ level

Effect of NAA on GFP Expression in the leaves of the seedlings

The change of the green fluorescence from GFP expression in the infected leaves showed the same trend as those of the biomass of tobacco seedlings (fig.4). Compared with the control, the expression of GFP in tobacco seedlings increased by 3.12%, 10.91%, 19.00%, 0.57%, and -1.92% after treatment with 0.1 mg/L, 0.2 mg/L, 0.4 mg/L, 0.8 mg/L and 1.6 mg/L of NAA, respectively.

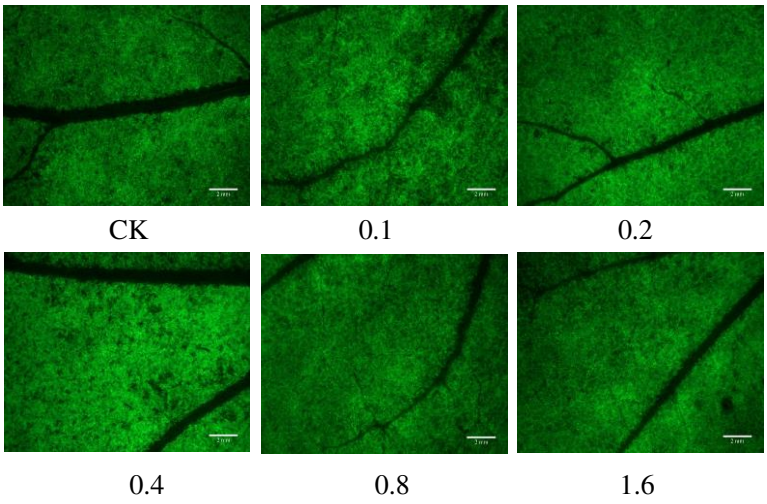


Fig. 3 The changes of GFP fluorescence expression in the seedlings treated with 0, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L NAA, respectively

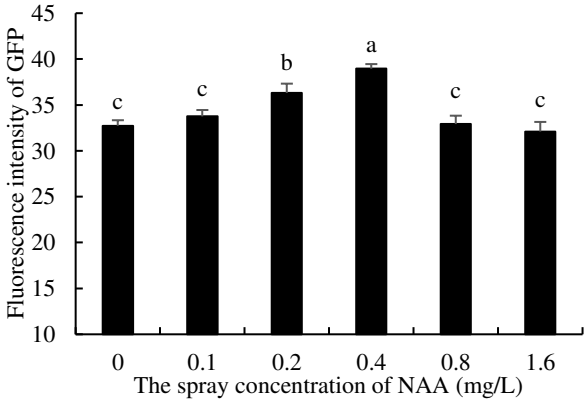


Fig. 4 The fluorescence expression of GFP in the seedlings

treated with 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/L NAA

Different lowercase letters indicated that there are significant differences in the same

parameter among the treatment with different concentrations at the $P < 0.05$ level

Effect of GA_3 on transient expression and growth of the seedlings

Effect of GA_3 on the growth of tobacco seedlings

It can be seen from fig.5 that, compared with the control (treatment with water), the treatment with 0.1 to 0.4 mg/L GA_3 significantly increased the fresh weight, dry weight, and leaf area of the seedlings. Furthermore, the biomass of the seedlings treated with 0.2 mg/L GA_3 was significantly higher than that of the seedlings treated at 0.4 mg/L GA_3. However, the 0.8 mg/L GA_3 failed to increase the fresh weight and leaf area of tobacco seedlings but increased the dry weight to some extent. When the concentration of GA_3 reached 1.6 mg/L, the biomass of the seedlings returned to a level similar to that in the controls.

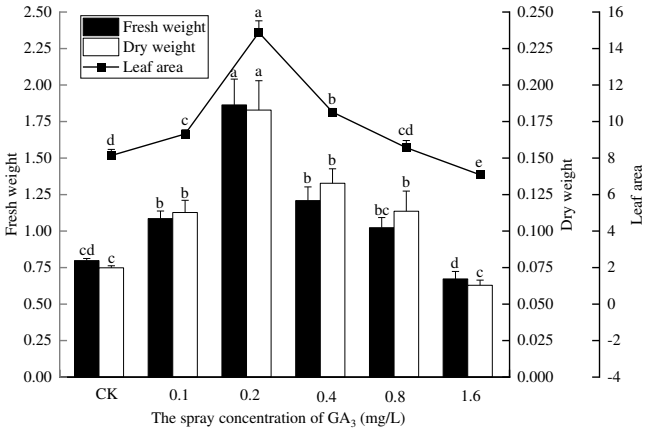


Fig. 5 Changes of fresh weight, dry weight, and leaf area

of the seedlings under different concentrations of GA_3

Different lowercase letters indicated that there are significant differences in the same parameter among the treatment with different concentrations at the $P < 0.05$ level

Effect of GA_3 on GFP Expression in tobacco seedlings

As shown in fig.7, with the increase of GA_3 spraying concentration, the expression of GFP increased at first and then decreased. Compared with the control, the expression of GFP in tobacco seedlings increased by 11.38%, 25.28%, 11.19%, -0.46% and -0.85% after treatment with 0.1 mg/L, 0.2 mg/L, 0.4 mg/L, 0.8 mg/L, and 1.6 mg/L GA_3, respectively.

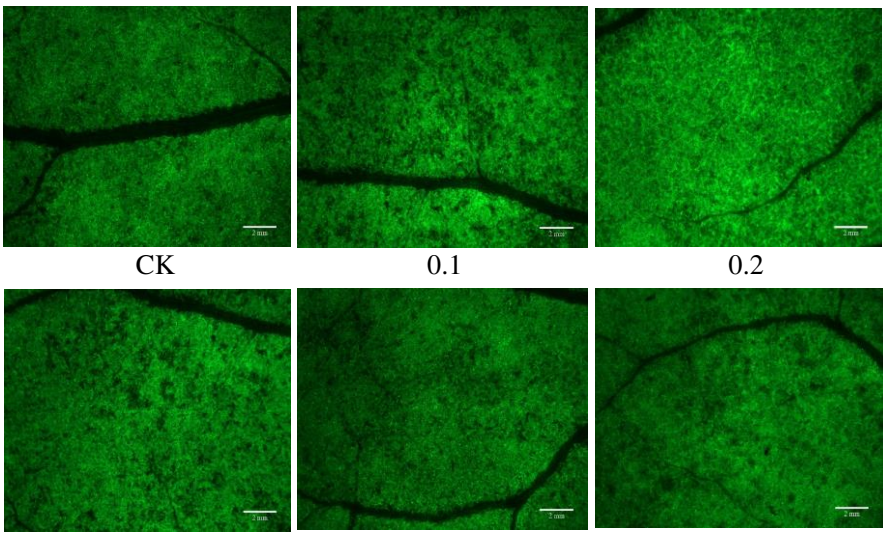


Fig. 6 Shows the changes in GFP fluorescence expression in the seedlings treated with 0, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L GA_3, respectively

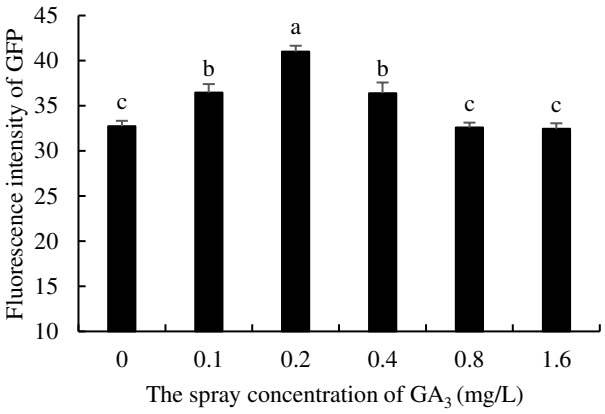


Fig. 7 The fluorescence expression of GFP in the seedlings treated with 0, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L GA_3

Different lowercase letters indicated that there are significant differences in the same parameter among the treatment with different concentrations at the $P < 0.05$ level

Effect of 6-BA on transient expression and growth of the seedlings

Effect of 6-BA on the growth of tobacco seedlings

It can be seen from fig.8 that treatment with 0.1 mg/L 6-BA did not significantly affect the biomass of the seedlings, compared to the control (treatment with water). 6-BA at 0.2 or 0.4 mg/L significantly increased the fresh weight, dry weight, and leaf area of tobacco seedlings, and the biomass of the seedlings treated with 0.2 mg/L 6-BA was significantly higher than that of the seedlings treated with 0.4 mg/L 6-BA. However, the 0.8 mg/L 6-BA failed to increase the fresh weight, dry weight, and leaf area of the seedlings. When the concentration of 6-BA reached 0.8 and 1.6 mg/L, the biomass of the seedlings returned to a level similar to that in the controls.

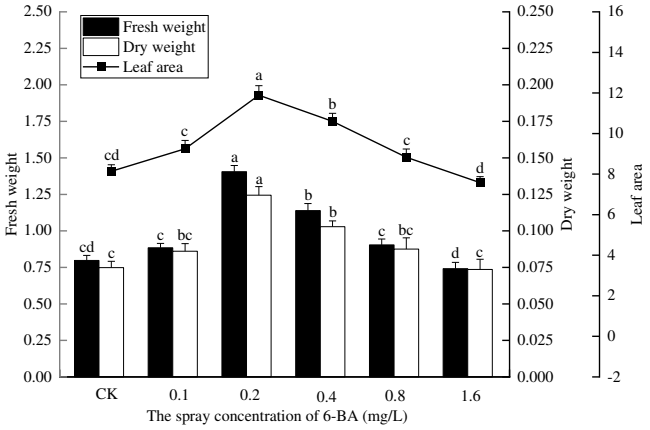


Fig. 8 Changes of fresh weight, dry weight, and leaf area of the seedlings under different concentrations of 6-BA treatment

Different lowercase letters indicated that there are significant differences in the same parameter among the treatment with different concentrations at the $P < 0.05$ level

Effect of 6-BA on GFP Expression in tobacco seedlings

As shown in fig.10, although the expression of GFP in infected leaves increased first and then decreased with the increase of 6-BA spraying concentration, the overall fluorescence expression in the leaves treated with 6-BA were lower than that of the control group. Compared with the control, the expression of GFP in tobacco seedlings decreased by 36.68%, 22.75%, 35.28%, 40.39%, and 54.30%, respectively, after treatment with 0.1 mg/L, 0.2 mg/L, 0.4 mg/L, 0.8 mg/L and 1.6 mg/L 6-BA.

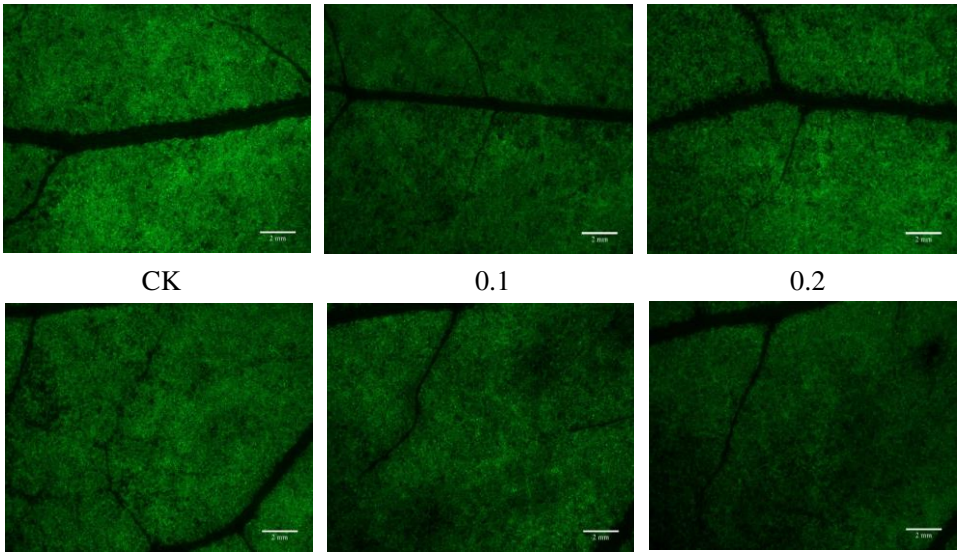


Fig. 9 The changes in GFP fluorescence expression in the seedlings treated with 0, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L 6-BA, respectively

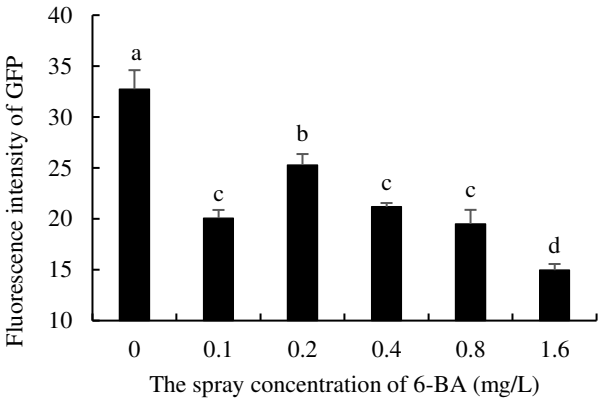


Fig. 10 The fluorescence expression of GFP in the seedlings treated with 0, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L 6-BA

Different lowercase letters indicated that there are significant differences in the same parameter among the treatment with different concentrations at the $P < 0.05$ level

Discussion

In the process of *Agrobacterium*-mediated transient expression systems, environmental control of plant

242 incubation conditions is crucial to enhance recombinant protein production (Fujiuchi et al., 2016;
243 Fujiuchi et al., 2014; Matsuda et al., 2017; Fujiuchi et al., 2017). Exogenous application of some
244 chemical components that have the potential to increase the recombinant protein production could be
245 more convenient and most easily controlled, compared to the control of light, temperate, and humidity.
246 It is well known that NAA, GA_3, and 6-BA are commonly used to enhance plant growth by promoting
247 cell elongation and expansion, or accelerating cell division. However, whether these three kinds of
248 PGRs can influence plant transient expression is still unknown.
249 It was not surprising that NAA, GA_3, 6-BA at moderate concentration enhanced the plant growth. In
250 detail, for the enhancement of the growth, the most effective concentration of these three different
251 PGRs is different. Among them, 0.4 mg/L NAA treatment led to the most obvious increase in the
252 biomass of the seedlings, while GA_3 and 6-BA at 0.2 mg/L led to the most obvious increase in the
253 biomass. The discrepancy may originate from the difference in regulation mechanism of plant growth
254 by these three PGRs. In conclusion, an optimal concentration of PGRs for plant growth is needed.
255 Excess low or high concentration would have no obvious effect on plant growth.
256 The aim of this study is to evaluate the effects of NAA, GA_3, 6-BA on transient expression. The results
257 showed that NAA, GA_3 at moderate concentration can enhance the level of transient expression, while
258 the 6-BA decreased the level of transient expression. In detail, the most enhanced level of transient
259 expression by NAA and GA_3 and their corresponding concentration were different. Among the

concentration of NAA used, 0.4 mg/L NAA led to the largest enhancement of transient expression of GFP (transient expression of GFP was enhanced by 19.00%). For GA_3, the largest enhancement of transient expression of GFP was achieved by 0.2 mg/L GA_3 (transient expression of GFP was enhanced by 25.28%). And, it seems that there was a certain correlation between the transient expression efficiency and growth among the seedlings treated with either NAA or GA_3 (from Fig.11). However, the 6-BA treatment decreased the level of transient expression of GFP, although it can enhance plant growth at a moderate concentration (0.2-0.4 mg/L).

The mechanism for the effects of these PGRs on the transient expression is unclear. Previous work reported that fungal cytokinin can weaken the host's defense and be beneficial to the growth of pathogens (Shen et al. 2018) and auxin can inhibit plant defense response (Depuydt et al. 2009), although whether the 6-BA can change the resistance of plants to pathogen has not been reported yet. Thus, it is possible that PGRs could affect the transient expression efficiency via affecting the resistance of the plant to *Agrobacterium*. However, in fact, it is difficult to obtain a clear mechanism for the effects of different PGRs on the level of transient expression. On one hand, the *Agrobacterium*-mediated transient expression is a complex process involving a series of biological events, including *Agrobacterium tumefaciens* infection, T-DNA transfer from *Agrobacterium tumefaciens*, protein biosynthesis, and its accumulation in leaf tissue (Gelvin 2003; Jamal et al. 2009; Matsuda et al. 2018). On the other hand, the effects of PGRs on plants are also very complex. Besides the possible effects

of PGRs on plant resistance and growth, it has been reported that PGRs can influence some physiological processes that could be related to the biological events of *Agrobacterium*-mediated transient expression (Shin et al. 2003; Nardi et al. 2014; Thirukkumaran et al. 2009; Mockaitis and Estelle 2008).

Regardless of how complex the mechanisms for the effects of these PGRs on the transient expression, the aim of the present work is to present whether the PGRs could be used to enhance the efficiency of *Agrobacterium*-mediated transient expression systems. In the current works about improving the efficiency of transient gene expression in plants, much attention is focused on the optimization of the vector by molecular biological methods. And, some works also made attempts to enhance the efficiency of transient gene expression by modifying external factors, mainly including light, temperature, and humidity. However, it is difficult or high-cost to control temperature, humidity, and illumination when transient gene expression is utilized to produce pharmaceutical-grade recombinant proteins. In comparison, PGRs can be easily applied and cost-efficient if they can be effective to increase recombinant protein yields from transient gene expression in plants. And, there are no security concerns when PGRs are used. As demonstrated by the present work, appropriate PGRs at moderate concentration could be a benefit for the yield of foreign protein and plant growth. Of course, further work is needed to evaluate the effect of PGRs on the production of pharmaceutical protein from *Agrobacterium*-mediated transient expression system in plants.

In conclusion, appropriate PGRs can enhance the efficiency of transient gene expression. Thus, it has the potential to be considered as the exogenous component to be applied in recombinant protein production by plant-based transient expression systems.

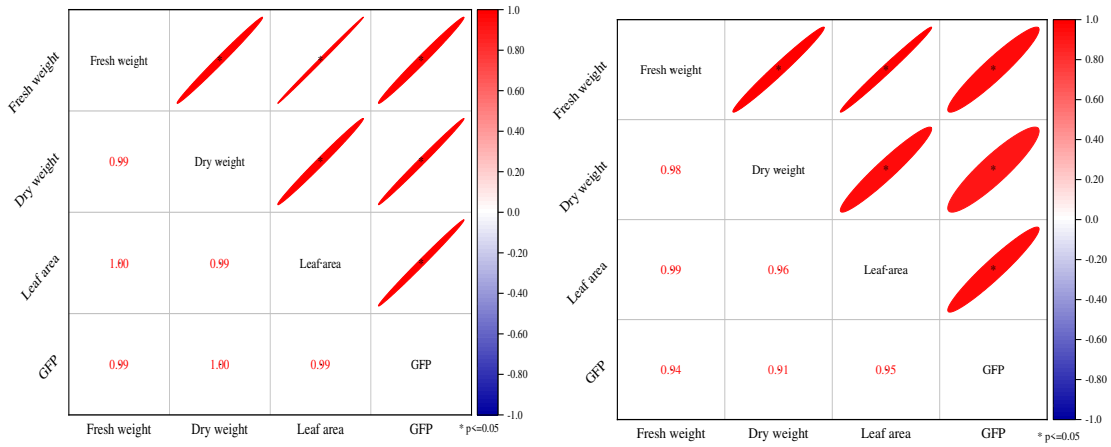


Fig. 11 The correlation between fresh weight, dry weight, leaf area, and GFP expression of the seedlings under 0.4 and 0.2 mg/L treatments of NAA and GA_3, respectively

Abbreviations

BeYDV: bean yellow dwarf virus; GFP: green fluorescent protein; NAA: α-naphthalene acetic acid; GA_3: gibberellins; 6-BA: 6-benzyladenine; PGRs: plant growth regulators; YEB: *Agrobacterium* rhizogene medium; ssDNA: single-stranded DNA.

Authors' contributions

YL and MS performed the core experiments and wrote the original manuscript draft. XW and YJZ performed the data analysis using Photoshop CS5, Image J, and IBM SPSS Statistics 19. XWD performed the green fluorescence intensity determination experiments. LYJ and HLP provided the

resources and supervision. HQF conceptualized, administered, reviewed, and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors approved the consent for publishing the manuscript.

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