# Improved the *Agrobacterium tumefaciens*-mediated transformation of cucumber by a modified the using of antibiotics and acetosyringone

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## Abstract

**Background:** Cucumber (*Cucumis sativus*) is one of the most important vegetable crops in the world. As conventional breeding of cucumber is very challenging, genetic engineering is an alternative option to introduce important traits such as enhanced stress resistance and nutritional value. However, the efficiency of the transformation system depends on genotypes, transformation conditions, selection agents, etc. This study aims to speed up the process of *Agrobacterium*-mediated transformation of cucumber. '*Xintai mici*', a very popular and typical north China-type cucumber variety, was transformed with *Agrobacterium* GV3101. The strain carried pCAMBIA2300s plasmid, a double vector with the marker gene of neomycin phosphotransferase II (*npt* II).

**Results:** The research results indicated that cefotaxime sodium was suitable for inhibiting

*Agrobacterium* in the stage of screening and bud elongation. Timentin was best used during rooting stage. Furthermore, 25 mg/L kanamycin was used in the early stage of screening and increased to 50 mg/L for further screening. At the bud elongation and rooting stage, 75 and 100 mg/L kanamycin was used respectively to improve the screening efficiency. In order to obtain the highest regeneration frequency of resistant buds, 50, 150, and 100 μM acetosyringone were added in the pre-culture medium, infection solution, and co-culture medium respectively. To confirm the presence of the transgenes, DNA from *npt* II transgenic cucumber plants was analyzed by polymerase chain reaction after transplanting resistant regenerated plants.

**Conclusions:** We finally achieved an 8.1% conversion, which was among the highest values reported until date using cucumber '*Xintai mici*'. Thus an effective protocol for *Agrobacterium tumefaciens*-mediated genetic transformation of cucumber was optimized.

**Keywords:** Cucumber, *Agrobacterium*, Kanamycin, Bacteriostatic antibiotics, Acetosyringone, Genetic transformation

## Background

Cucumber (*Cucumis sativus*) is one of the most important vegetable crops widely grown in the world. The studies on gene function and genetic breeding of cucumber have been concerned world widely[1, 2]. Abnormal expressions of genes in transgenic cucumber plants can help us to engineer and select more robust crop species, such as resistance to biotic and abiotic stress, fruit quality improvement[3], growth and development[4, 5]. Since the advent of cucumber tissue

culture and genetic transformation technology[6, 7], researchers have made a lot of achievements in the transformation of genes through improvement and optimization of transformation methods. In present, *Agrobacterium tumefaciens*-mediated transformation system of cucumber is still one of the most mature and popular genetic transformation methods[8]. However, the genetic transformation efficiency of cucumber is still low. The key factors affecting the infection efficiency of *Agrobacterium* include the type of explant[9], *Agrobacterium* species[10], exogenous hormone[11, 12], selection agent[13], *Agrobacterium* inhibitors[14] and phenols[15], etc. In addition, the cutting ways of explants[16] and the mode of infection, such as vacuum infiltration treatment[13, 15], also have an impacts on the transformation of cucumber in varying degrees.

In the selective culture stage of cucumber genetic transformation, selection antibiotics and antimicrobial antibiotics are often used together[17]. Kanamycin, hygromycin and glyphosate are used for selection markers commonly[13]. It is generally believed that the resistance of cucumber explants to selection antibiotics is closely related to its genotype and culture stages. Instead, antibiotics that have little effect on explants and certain inhibition effect on *Agrobacterium* are often selected and used[17, 18], such as cefotaxime sodium, carbenicillin, and timentin[15]. However, they are often used at a high concentration, which also have a certain impacts on the regeneration and growth of explants[19]. Many studies found that phenolic had a significant effect on the regeneration frequency of resistant buds, which was added to the pre-culture, infection and co-culture stages of genetic transformation[15, 20]. It is widely recognized that the phenolic, such as acetosyringone, can activate the *vir* gene of *Agrobacterium* and promote the introduction of

foreign genes into the plant genome, and then the efficiency of genetic transformation is improved[21]. However, acetosyringone is often dissolved in toxic organic solvents or has toxic effects in high concentrations and interacts with infection modes and other transform conditions[22]. Therefore, the specific addition stages and concentrations of acetosyringone were different in the existing reports[1].

The unconformity between the infection site of *Agrobacterium* and the regeneration site of explants is the most important reason for the low efficiency of the genetic transformation of cucumber. And the inconsistent sites are affected by various conditions and factors[1, 23]. Besides, the selection and the concentration of antibiotics are the main factors affecting the regeneration of positive buds[14]. However, these parameters were different in various literatures and also lack of enough details in the existing studies. All of these reasons leaded to browning, *Agrobacterium* pollution, vitrification shoots, low efficiency of genetic transformation[24], gene expression and genetic instability, which still perplex researchers. In order to improve the efficiency of genetic transformation, the effects of different concentrations of kanamycin on the regeneration of cotyledonary nodes by applying it in different transformation periods were analyzed in the present study. Meanwhile the inhibitory effects of three different antibiotics on *Agrobacterium* and the effects on regenerated buds were evaluated, and the effects of acetosyringone on the regeneration frequency of resistant buds in different stages of cucumber genetic transformation were researched. This study hopes to provide a reference for the future researches of cucumber transgenic.

# Results

## Effects of kanamycin on regeneration frequency and browning of explants

The untransformed explants were placed in the mediums with different kanamycin concentrations for 28 d (Fig. 1-a). The regeneration frequency of buds and browning level showed an opposite trend with the increase of kanamycin concentration. The regeneration frequency of 0 mg/L treatment was 90.0% and significantly higher than that of any other treatments. The prominent differences were observed between 25, 75, and 100 mg/L treatments. However, the regeneration frequencies of 50, 75, and 100 mg/L treatments was not notable difference. Browning began to appear at 50 mg/L treatment and reached the peak at 100 mg/L treatment (90.0%). Except for 100 mg/L treatment, there was no obvious difference among other treatments (Fig. 1-b).

## Effects of bacteriostatic antibiotics on *Agrobacterium* and explants

The explants were cultured in the selective medium containing 300 mg/L of cefotaxime sodium, carbenicillin, and timentin respectively for 28 d (Fig. 2-a). The experiment identified that 300 mg/L of the three antibiotics could inhibit the growth of the *Agrobacterium* on the explants. Cefotaxime sodium treatment had the highest resistant buds frequency (76.7%) and shared the same level of difference with timentin treatment. The lowest resistant buds frequency (43.3%) came from carbenicillin treatment and had no significant difference with timentin treatment (Fig. 2-b). The part c of Fig. 2 showed the growth of *Agrobacterium* on different types of antibiotics. When the culture time researched 96 h, the highest diameter of inhibition zone was in timentin treatment (6.4 cm). On the contrary, carbenicillin treatment was the lowest (5.6 cm). Significant

differences were seen in all treatments. Another interesting finding was at the culture time of 30 d, the maximum diameter of inhibition zone (5.8 cm) in response to the timentin treatment was observed. And the result was similar with timentin and cefotaxime sodium treatments. However, the diameter of inhibition zone of carbenicillin treatment decreased significantly and reached to the lowest level (4.4 cm) over time (Fig. 2-d). In another experiment, uninfected explants were cultured with different concentrations of cefotaxime for 28 d (Fig. 2-e). With the increase of cefotaxime sodium concentration, the number of regenerated buds in each explant decreased from 7.1 to 2.7, which almost reduced 4 times. Compared with 0 mg/L treatment, the 100 mg/L treatment was not significantly altered the numbers of regeneration buds. The number of regeneration buds of 0 and 100 mg/L treatments were significantly higher than that of any other treatments. There was no significant difference among 200, 300 and 400 mg/L treatments (Fig. 2-f).

**Effects of acetosyringone on regeneration of resistant buds**

Different concentrations of acetosyringone were added respectively to four important stages of genetic transformation respectively, the pre-cultivation (Fig. 3-a), infection (Fig. 3-b), co-cultivation (Fig. 3-c), and selective culture stages (Fig. 3-d). The regeneration frequencies of resistant buds were counted respectively after 28 d and shown as follows.

In the test of adding acetosyringone in the pre-cultivation stage, the frequency of resistant buds increased initially and then decreased with the raise of acetosyringone concentration. 50 μM treatment had the highest frequency of resistant buds (53.3%) and which was significantly higher

than that of 100, 200, and 400 μM treatments. Whereas, acetosyringone greater than or equal to 100 μM showed severe inhibition to the frequency of resistant buds. There was no notable difference between 0 and 50 μM treatments (Fig. 3-e). As shown in Fig. 3-f, the final frequency of resistant buds increased first and then decreased with the raise of acetosyringone concentration in the process of *Agrobacterium* inoculum. The regeneration frequency of resistant buds reached the peak (53.3%) under 150 μM treatment, which was significantly lowe than 200 μM treatment (20.0%). Besides 150 μM treatment, no significant difference was found between any other treatments. From the Fig. 3-g we could know that the frequency of resistant buds increased first and then decreased with the raise of acetosyringone concentration in the co-cultivation stage. 100 μM treatment had the highest regeneration frequency of resistant buds (80.0%), which was at the same level of difference with 50 and 200 μM treatments and comparatively significantly higher than that of the 0 and 400 μM treatments. The lowest regeneration frequency of resistant buds was in 0 and 400 μM treatments (33.3%), which had no obvious difference compared with 50 and 200 μM treatments. The explants grew in a screening medium with additional acetosyringone, and the results was set out in Fig. 3-h. As promoting the concentration of acetosyringone from 0 to 200 μM, the frequency of resistant buds decreased from 53.3% to 23.3%. The frequencies of resistant buds of 0, 50, and 100 μM treatments were at the same difference level, and 50, 100, 150, and 200 μM treatments were sharing another difference level. Taken together, these results suggested that extra acetosyringone could increase the regeneration frequency of resistant buds while in the early stages of transformation, but not after it was been screening (Fig. 3).

### Polymerase chain reaction (PCR) analysis

The regenerated plants of cucumber were domesticated. And the total DNA of the ninth tender leaf was extracted. The primers of report gene *npt* II were used to identify the transformed plants (Fig. 4-a), and a 480 bp product was amplified, which was the same as the positive control. The primers of the *Agrobacterium* genome were used to eliminate the contamination of plants from *Agrobacterium*, and the total DNA of regenerated plants was not amplified except for lane 12, 17, and 18 (Fig. 4-b). DNA of wide-type plant and negative control were not amplified (Fig. 4).

## Discussion

This study was conducted to establish an *Agrobacterium tumefaciens*-mediated transformation system for cucumber. We evaluated the optimal dosage of kanamycin and various antimicrobial antibiotics by observing the growth of explants and *Agrobacterium* in different concentrations. The addition amount of acetosyringone was changed when applied to different culture stages. Then the optimized regeneration protocol was adapted to transformation for cucumber.

### Effects of kanamycin on explants

Kanamycin has a great inhibitory effect on untransformed explants, especially the growth of root[25]. Therefore, kanamycin was widely used as a selection marker with successful results[26, 27]. The screening concentration of different cucumber varieties needed to be explored owing to the different sensitivity to kanamycin. As shown in Fig. 1, the regeneration of the buds was inhibited completely at 75 mg/L kanamycin, but the explants began to brown at the same time. In

order not to affect the regeneration of the delicate explants, 25 mg/L kanamycin was used after co-culture and then raised to 50 mg/L for further screening without browning. In addition, the concentration of kanamycin could be increased to 75 and 100 mg/L to prevent the emergence of false-positive plants[28] at buds elongation and rooting culture stages. The dynamic concentration of kanamycin (50–100 mg/L) accorded with the relevant reports[29, 30].

### Effects of bacteriostatic antibiotics on *Agrobacterium* and explants

Different strains of *Agrobacterium* had different sensitivities to antibiotics[31]. The specific effect of the antimicrobial antibiotics was not reported in the cucumber transformation. Therefore, it is very important to select the antibiotics which can effectively inhibit the pollution of the *Agrobacterium* GV3101 and have little effect on cucumber regeneration buds simultaneously. There were significant differences in the effects of three commonly used antimicrobial antibiotics (cefotaxime sodium, carbenicillin, and timentin) on the explants and *Agrobacterium*. This data demonstrated that the highest regeneration frequency of resistant buds could be obtained by using cefotaxime sodium (Fig. 2-a and b). It might due to the related chemical structures with auxin which could interact with others in the culture medium[32]. Timentin had the best antibacterial effect and the longest duration than others (Fig. 2-c and d) for its highly resistant to β-lactamases produced by bacteria[33] but was not as cheap and common as cefotaxime sodium in practical use. Besides, we found that the medium containing cefotaxime sodium (Fig. 5-II) would turn yellow gradually within 2 weeks in buds elongation culture stage, which could not be seen in the medium with timentin (Fig. 5-I). It was reported that the yellowing medium containing harmful substances

was caused by the accumulation of o-quinones through enzymatic browning[34]. On the other hand, the toxic effect of cefotaxime sodium on the shoot rooting had been reported[17]. In this sense, cefotaxime sodium was more suitable for cucumber genetic transformation and could be added in the medium in the selective and buds elongation culture stages, while timentin could be added in rooting culture stage because of its long lasting effect[35]. Besides, carbenicillin was unsuitable for the genetic transformation of cucumber '*Xintai mici*'. The effect of cefotaxime sodium on the explants further determined indicated that 100 mg/L concentration of cefotaxime sodium had little effect on explants, while 500 mg/L showed significant inhibition (Fig. 2-e and f). The suitable concentration of cefotaxime sodium was 200–400 mg/L, which could be adjusted according to the extent of pollution from *Agrobacterium*.

## Effects of acetosyringone on the regeneration of resistant buds

Yadav et al. indicated that twelve low molecular weight phenolic compounds and salicylic acid were the main substances secreted after the explants of chick-pea (*Cicer arietinum* L.) were injured, and polyphenol oxidase was activated to oxidize phenols[34]. These led to the decrease of phenol and the increase of o-quinones gradually with time, which was one of the factors that resulted in the difficulty of T-DNA transport. Also, secretions such as salicylic acid and gallic acid also inhibited the growth and transformation of *Agrobacterium*. In many studies, the transformation efficiency of cucumber was improved by adding additional phenol, such as acetosyringone[20, 27]. It could also be achieved by inhibiting the oxidation of phenols, like adding antioxidants, such as α-Caprylic acid, L-Cystine, dithiothreitol, and $Na_2S_2O_3$[34]. The

study that adding different contents of acetosyringone in four key steps of genetic transformation showed that the content of acetosyringone had a significant effect on the regeneration of resistant buds. The analyzed results showed that the addition of acetosyringone had a significant effect on the regeneration of resistant buds in different stages (Fig. 3). In the pre-cultivation stage, only 50 μM acetosyringone was needed to improve the regeneration frequency of resistant buds. The regeneration frequency of resistant buds would be lower than the control level (0 μM treatment) with too high acetosyringone concentration. The best concentration of acetosyringone in the infection liquid of *Agrobacterium* was 150 mg/L. And the high concentration would make the regeneration frequency of resistant buds drop sharply. Similarly, the optimum concentration of acetosyringone in the co-culture medium was 100 mg/L, and the highest regeneration frequency of resistant buds was 80.0%. In the stage of selective culture, the extra acetosyringone did not help increase the regeneration frequency of resistant buds. On the contrary, the presence of acetosyringone decreased the differentiation resistance of explants. Through these researches, we had successfully determine the content of acetosyringone in the process of cucumber genetic transformation, which was the basis for improving the transgenic efficiency of cucumber '*Xintai mici*'.

## Conclusions

In recent years, although new genetic transformation methods had been reported, such as nanoparticles-mediated genetic transformation[36]. New technologies like CRISPR/Cas9[1, 37] and selection markers with no antibiotic[38] were also applied to the genetic transformation of

cucumber. The *Agrobacterium tumefaciens*-mediated transformation system of cucumber is still one of the most concerned transformation methods, and the improvement scheme is still the focus. But cucumber is still one of the most difficult species for transformation, although it has been carried out for 39 years[7]. The highest transformation efficiency of cucumber was 26%[2], while the lowest was only 0.1%, and mostly between 1%–10%[2]. Moreover, there were few reports about the transformation of cucumber '*Xintai mici*'[39].

The transformation method reported here is the modification and improvement scheme of the previous reports in cucumber. The complete transformation method was used in the study and the main steps of which were shown in Fig. 5. We increased the infection depth through the vacuum system. And we set a gradient concentration of kanamycin to prevent the damage of tender plants and the emergence of false-positive and chimeric plants. We used three antimicrobial antibiotics in different stages by comparing their effects on the growth of *Agrobacterium* GV3101 and explants. By changing the usage of acetosyringone in the important genetic transformation stages, we improved the regeneration frequency of resistant buds by increasing the content of phenolics. We successfully obtained the transgenic plants from cotyledonary nodes of cucumber '*Xintai mici*' after 3-month of application of the improved genetic transformation system (Fig. 5). From 223 explants, 134 resistant buds were regenerated, and 42 rooting regenerated plants were obtained. At last, 18 plants were identified as positive. The positive rate was 42.8%, and the transgenic efficiency was 8.1%, which had been improved comparing with other reports[39]. This project was undertaken to improve *Agrobacterium*-mediated transformation of cucumber with similar

genetic background with '*Xintai mici*' and laid a foundation for other gene transformation work.

# Materials and Methods

## Plant materials and media

The seeds of cucumber '*Xintai mici*' (a north China-type cucumber variety) were soaked for 2–3 h in water, whose seed coat were peeled. The unclad seeds were dipped in 75% ethanol for 1 min and 15% sodium hypochlorite for 15 min, then were rinsed 4 times in sterile distilled water. The sterilized seeds were germinated in the dark at 28℃ for 48 h and light for 24 h with the medium-I (2.21 g/L M519 + 15 g/L sucrose + 2.5 g/L phytagel, pH=6.8). Cotyledonary nodes were first cut in half, 2 mm hypocotyls were retained, the distal 2/3 parts and growth point were removed. Cotyledonary nodes were cultured in the medium-II (4.43 g/L M519 + 30 g/L sucrose + 2.5 g/L phytagel + 0.5 mg/L 6-benzylaminopurine + 1.0 mg/L abscisic acid + 1.0 mg/L $AgNO_3$, pH=6.8) with varying concentrations (0, 50, 100, 150, and 200 μM) of acetosyringone in the dark at 28℃ for 24 h.

## *Agrobacterium* strain and vector

The *Agrobacterium* strain GV3101 was used for transformation. The binary vector was pCAMBIA2300s, including the neomycin phosphotransferase II (*npt* II) selection marker, driven by the CaMV-35S promoter. The *Agrobacterium* was resuscitated in Luria-Bertani (LB)-I medium (5 g/L yeast extract + 10 g/L tryptone + 10 g/L NaCl + 15 g/L agar, pH=5.8) with 50 mg/L kanamycin, 25 mg/L rifampicin at 28℃ until single colonies appearing. The *Agrobacterium* single

colonies were added to 1mL of LB II (5 g/L yeast extract + 10 g/L tryptone + 10 g/L NaCl, pH=5.8) with 50 mg/L kanamycin, 25 mg/L rifampicin at 28℃ until turbid. Then the *Agrobacterium* was cultured with 100 mL of LB II containing 50 mg/L kanamycin, 25 mg/L rifampicin at 28℃ until optical density at 600 nm ($OD_{600}$) of 0.6–0.8 was achieved. The *Agrobacterium* culture was centrifuged and resuspended in the medium-III (2.21 g/L M519 + 15 g/L sucrose, pH=6.8), and the final concentration of the *Agrobacterium* (Measured by $OD_{600}$) was adjusted to 0.2–0.3. Varying concentrations (0, 50, 100, 150, and 200 μM) of acetosyringone were added to the medium-III. Before inoculation, the resuspended *Agrobacterium* inoculum was shaken for the induction of *vir* genes at 28℃ for 1 h.

## Effects of kanamycin and bacteriostatic antibiotics on *Agrobacterium* and explants

Explants that were not being exposed to *Agrobacterium* were placed on the medium-II. Varying concentrations of kanamycin (0, 25, 50, 75, and 100 mg/L) and cefotaxime sodium (0, 100, 200, 300, 400, and 500 mg/L) were added in the medium-II respectively. Petri dishes were placed in the tissue culture room, 28℃, 4000 Lx, 16 h/d. The medium was changed every 2 weeks for 28 d. 100 μL of the *Agrobacterium* ($OD_{600}$=0.7) was added in the LB-I medium. A piece of 6 mm diameter sterile filter paper with 0.5 mg of bacteriostatic antibiotics (cefotaxime sodium, carbenicillin, and timentin) was placed in the center. Petri dishes were placed in the 28℃ bacteria incubators for 96 h and 30 d.

## Inoculation, co-cultivation, screening, and regeneration

Explants were immersed in *Agrobacterium* inoculum in sterile Erlenmeyer flasks with

breathable filter membranes. Erlenmeyer flasks were placed in a vacuum system, 0.094 MPa for 5 min. The vacuum was relieved slowly to prevent damage to explants caused by stress transients. The infected explants were cultured in the medium-II with a sterile filter paper in the dark at 28℃ for 48 h[40, 41]. Varying concentrations (0, 50, 100, 200, and 400 μM) of acetosyringone were added in the medium-II. After co-cultivation, explants were washed 5 times with sterilized distilled water. Explants were blotted dry on sterile filter paper. Then explants were transferred to the medium-II. 50 mg/L kanamycin, the varying concentrations of acetosyringone (0, 50, 100, 200, and 400 μM) and bacteriostatic antibiotics (cefotaxime sodium, carbenicillin, and timentin, 300 mg/L) were added in the medium-II respectively. Petri dishes were placed in the tissue culture room, 28℃, 4000 Lx, 16 h/d. The medium was changed every 2 weeks until 2-cm-high regenerating buds were grown.

Resistant regeneration buds were cut off and transferred to the medium-IV (4.43 g/L M519 + 30 g/L sucrose + 2.5 g/L phytagel + 0.2 mg/L 6-benzylaminopurine + 1.0 mg/L $AgNO_3$, pH=6.8) containing 75 mg/L kanamycin and 300 mg/L cefotaxime sodium or timentin to grow for 2 weeks. Then the resistant regeneration buds were transferred to the medium-V (2.21 g/L M519 + 15 g/L sucrose + 2.5 g/L phytagel + 1.0 mg/L $AgNO_3$, pH=6.8) containing 100 mg/L kanamycin and 300 mg/L timentin to induce rooting for 2 weeks. The regeneration plants with flourishing roots were transferred into the matrix ($V_{Peat}$: $V_{Perlite}$=1: 1) and domesticated in an artificial climate chamber (Day: 28℃, 6000Lx, 16 h; Night: 18℃, 8 h; Relative humidity: 65%)[42]. Each regeneration cucumber was covered with cling film to maintain humidity for 1 week. Then the cucumber plants

were managed by normal water and fertilizer[43].

## DNA isolation and PCR analysis

Cucumber '*Xintai mici*' was genetically modified with the improved genetic transformation system. After obtaining regenerated plants, the ninth new leaf of the cucumber regeneration plants was removed, quick frozen with liquid nitrogen, and their total DNA was extracted by CTAB method[44]. PCR was used to confirm the presence of the transgene in primary transformants. PCR reactions were carried out in a 20 μL volume containing 2 μL of 10 ×PCR Buffer, 200 μM of each dNTP, 0.4 U r*Taq* DNA polymerase, 100 ng template DNA, 1 μM of each primer. The primer sequences were *npt* II Forward 5'-TCGGCTATGACTGGGCACAACAGA-3' and *npt* II Reserve 5'-AAGAAGGCGATAGAAGGCGATGCCT-3', yielding an amplification product of 480 bp. Excluding the *Agrobacterium* genome primers were *HrcA* Forward 5'-CATCGTCGAAGGTTATCTCGATACG-3' and *HrcA* Reserve 5'-TATAATCGACCATCGGTACGATACG-3'[15], yielding an amplification product of 800 bp. PCR amplification was performed as follows: 94℃ for 3 min, 35 cycles of 94℃ for 30 s, 58℃ for 30 s, 72℃ for 1min, followed by a final extension of 72℃ for 10 min. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

All authors agreed to publish this manuscript.

## Availability of data and materials

All data generated or analyzed during this study are available in this published article.

## Competing interests

The authors declare that they have no competing interests.

## Funding

This work was financially supported by the Natural Science Foundation of Zhejiang Province of China (LY18C150004, LY18C150003, LY15C150006), and the National Natural Science Foundation of China (31101539 and 31201658).

## Authors' contributions

C. D. and H. F. conceived and designed the study. L. C., C. L., and Y. Y. S. performed the experiments. L. C. wrote the paper with inputs from all authors.

## Acknowledgments

We are grateful for helps from the Zhejiang Provincial Key Laboratory of Agricultural Product Quality Improvement Technology in Zhejiang A&F University.

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