STUDY ON PLANT REGENERATION AND FUNGAL DISEASE RESISTANT GENE TRANSFORMATION FOR VIETNAMESE GINGER (Zingiber officinale Rosc.) VIA Agrobacterium - MEDIATED METHOD

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SUMMARY

Ginger (Zingiber officinale Rosc.) has been used as valuable spice and medicine for a long time in South Asia. Ginger and other species in the Zingiber genus have known as a recalcitrant group for tissue culture and transformation. In this study, we assessed the regeneration media for ginger plant regeneration from in vitro leaf sheaths. Results showed that the MS medium containing 1 mg/L thidiazuron gave the highest efficiency with 68.33% in regeneration rate and 24.63 shoots forming per regenerated sample in average. These results were extremely useful for the application on genetic transformation in ginger which is susceptible to fungal diseases. A plant defensin gene (Ca-AFP) was transferred into in vitro leaf sheaths of ginger simultaneously with β-glucuronidase (gus) reporter gene and hygromycin phosphotransferase (hpt) selectable marker gene, by using the Agrobacterium tumefaciens strains EHA105. After 10 weeks in selective media containing 4 mg/L hygromycin, the expression of gus gene was recorded in some regenerating shoots by histochemical GUS assay. By using PCR detecting method, the positive bands of Ca-AFP and hpt transgenes were observed in four lines of transformed shoots that regenerated in hygromycin-selective media within 20 weeks. The results of this study show the potential to create a new ginger variety that could improve the resistance to soft rot disease caused by Pythium aphanidermatum fungus in the future.

Keywords: Agrobacterium tumefaciens, plant defensin gene, regeneration, Zingiber officinale Rosc.

INTRODUCTION

The genus Zingiber is distributed in tropical and subtropical Asia and Far East Asia and includes about 150 species. Ginger (Zingiber officinale Rosc.) is one of essential spice crops and herbs that have cultivated and used in South Asia for a long time (Ravindran, Babu, 2005). The ginger rhizome contains bioactive compounds such as gingerol, shogaol, zingiberene, α-terpineol and linalool (Ravindran, Babu, 2005; Zuraida et al., 2016). However, ginger is susceptible to fungi and bacteria, especially Pythium aphanidermatum causing soft rot disease and it is easy for the pathogen to be widely transmitted. Therefore, after being infected by pathogen, extreme yield losses can occur in ginger cultivation (Ravindran, Babu, 2005). Because of those problems, development and propagation of disease-free or disease-highly-resistant ginger varieties are required with effective methods. Genetic transformation is one of solutions that have been successful on some main crops (cotton, maize, and soybean) to enhance the resistance to insects or diseases (ISAAA, 2017).

For ginger, callus and embryogenic callus could be induced from ex vitro buds, young leaves and anthers. Type of auxin and their concentration supplied to media have the main role on induction of somatic embryogenesis. Many types of auxin have been reported for their efficiency, such as 2,4-D, dicamba or NAA (Kackar et al., 1993; Ravindran, Babu, 2005). Combination of 2,4-D and BA, or NAA and kinetin/BA could also induce embryogenic callus from shoots (Jamil et al., 2007; Suma et al., 2008). However, the number of such reports are limited and it is difficult to apply these results for genetic transformation because the limit of ex vitro explants (Suma et al., 2008). In previous study on Vietnamese ginger, the combination of BA and 2,4 D could induced embryogenic callus and somatic embryo from transverse thin cell layer of ex vitro rhizome sprouts (Tran et al., 2010). However, ginger rhizomes are usually collected at the end of cultivation seasons. Thus, the in vitro aerial stem (including shoot tips meristem) has been used for plant regeneration instead of ex vitro sprouts. The use of 2,4 D was inefficient when the regeneration rate was lower than 10%. On the other hand, thidiazuron (TDZ) is one of the most active cytokinin, can induce adventitious shoots more effective than purine-type cytokinin in many species such as carnation (Lu et al., 1991; Nugent et al., 1991), and rose (Lu, 1993). TDZ also stimulates endogenous purine cytokinin synthesis and inhibits their degradation (Capelle et al., 1983; Thomas, Katterman, 1986). Therefore, when TDZ was applied on media, regeneration rate from in vitro aerial stem was around 70% (Tran et al., 2019). This is the potential result which need to be studied more detail.

Plant defensins are small, cysteine-rich peptides, abundantly present in plant kingdom, usually are found in stomatal and peripheral cells. They play important roles on primary effect on the invading pathogens, especially against fungal pathogens (Khan et al., 2019). Three-dimensional pattern of plant defensins are conserved with
one alpha helix and three antiparallel beta sheets. These folding patterns are stabilized by four or five disulfide bridges from conserved amino acid residues of cysteine (Khan et al., 2019). Ca-AFP gene from chickpea (Cicer arietinum) can encode a plant defensin protein of 74 amino acids. The results from aligning Ca-AFP protein’s sequence to those of other crops’ defensin proteins showed that Ca-AFP contains eight common cysteine residues, which include four disulfide bridges (Kumar et al., 2019). Furthermore, it was demonstrated that Ca-AFP protein has the antifungal function to Pythium aphanidermatum and Botrytis cinereal (Islam et al., 2007).

On genetic transformation in plants, selecting agent plays an important role on the screening of stable transgenic explants, and eliminate chimeric status. Hygromycin B, an antibiotic, is often used as the selecting agent. This is the second most frequently used antibiotic for selection after kanamycin (Miki, McHugh, 2004). So far, there is only one report that uses hygromycin with 40 mg/L in concentration to screen the transformants in Z. officinale cultivated in India (Musfir Mehaboob et al., 2019).

In this study, we developed a regeneration protocol for Z. officinale, a Vietnamese variety, by using young in vitrO leaf sheaths to create a stable source for genetic transformation of this species. Initially, Ca-AFP gene under the control of CaMV35S promoter was transferred into Z. officinale by Agrobacterium tumefaciens for an attempt to improve resistance to soft rot disease caused by Pythium aphanidermatum fungus.

**MATERIALS AND METHODS**

**Plant materials**

Sprouts forming in rhizome of cultivated ginger (Zingiber officinale) in the southern of Vietnam were collected and established in vitro as the description in our previous report (Tran et al., 2010). They were micro-propagated in solid Murashigae and Skoog (MS) medium supplemented with 3 mg/L BA, and used as in vitro source. They were sub-cultured every two-month and maintained at 25 ± 2°C with photoperiod of 8 hours light and 16 hours dark (Tran et al., 2010; 2019).

**Evaluation of shoot regeneration medium**

Leaf sheaths were cut from 1-month-old in vitro explants with approximate 5 mm in length and we investigated the effect of TDZ on shoot regeneration from leaf sheaths. MS medium was supplemented with different concentrations of TDZ (0.5, 1.0, and 1.5 mg/L). We also examined the efficiency of shoot regeneration in the presence of both 0.5 mg/L IBA and TDZ (in the above range of concentration). Samples were transferred to fresh media every two-week, at 25 ± 2°C with photoperiod of 8 hours light and 16 hours dark.

**Assessment of hygromycin concentrations transgenic shoot selection**

In this study we assessed the effect of hygromycin on the regeneration of shoots from in vitro leaf sheaths of Vietnamese Z. officinale cultured in optimal regeneration media. Concentrations of hygromycin in this assessment were in range 0, 2, 4, and 6 mg/L, and samples were sub-cultured every two-week.

**Agrobacterium strain, T-DNA construct and transformation**

Agrobacterium tumefaciens strain EHA105 harbors a T-DNA construct shown in Figure 1. A. tumefaciens strain was cultured in liquid LB medium supplemented with kanamycin and rifampicin by incubating at 28°C, and shaking at 200 rpm overnight. Before the infection, cultured A. tumefaciens was centrifuged at 3500 rpm for 10 minutes. Then, the pellet was resuspended in liquid ½ MS medium supplemented with 200 µM acetosyringone, and the optical density (OD600) was in the range of 0.8~1.0. The leaf sheath segments were soaked in A. tumefaciens solution for 20 minutes. After that, the excessive bacteria in infected samples was absorbed by aseptic filter papers. Then, infected samples were co-cultured in basal MS medium supplemented with 200 µM acetosyringone in the dark at 28°C for 3 days.

**Selection of transgenic shoots**

After co-culture period and washing re-grew A. tumefaciens, samples were placed on shoot-regeneration medium supplemented with 4 mg/L hygromycin to select the transformed line and antibiotic (cefotaxime and augmentin) to eliminate A. tumefaciens. The sub-cultivation was performed in selective medium every two-week until getting the regenerating shoots.
Histochemical Gus assay

In order to evaluate the expression of gus gene in infected ginger leaf sheaths, histochemical Gus assay was performed as the Jefferson's protocol (Jefferson, 1987). X-Gluc was dissolved in 100 mM sodium phosphate buffer (pH 7.0) containing 10 mM EDTA, 0.5 mM K$_4$[Fe(CN)$_6$], 0.5 mM K$_3$[Fe(CN)$_6$], and 0.1% (v/v) Triton™ X-100 to reach the 1 mM concentration. Samples were incubated on X-Gluc solution at 37° C overnight. Then, samples were soaked in 70% ethanol for 4 hours to remove chlorophyll (Jefferson, 1987).

PCR analysis

Leaf blades of hygromycin-resistant shoots regenerating in selective medium were collected and genomic DNA were extracted by using DNeasy® Plant Mini Kit (QIAGEN, Germany). PCR amplification was carried out with Swift™ Maxi Thermal Cycler (ESCO, Singapore) according to the following step-cycle program: (1) pre-incubation at 95° C for 5 minutes, follow by 30 cycles of denature at 95° C for 60 seconds, and annealing at 55° C for 60 seconds and extension at 72° C for 2 minutes each cycle for hpt gene; and (2) pre-incubation at 95° C for 5 minutes, follow by 35 cycles of denature at 95° C for 90 seconds, and annealing at 65° C for 90 seconds and extension at 72° C for 2 minutes each cycle for Ca-AFP gene. The primer pairs for PCR were the following: 5'- AGCTGGCAGATTTCTACAA -3' and 5'- ATCGCTCGTCCAGTAGT -3' for hpt gene and 5'- CGCGGATCCATGGCGAGGTGTGAGAATTTGGCT -3 and 5'- TGCTCTAGATGGCAGGTGAGAATTTGGCT -3' for Ca-AFP gene (El-Siddig et al., 2009; Nguyen et al., 2012).

RESULTS AND DISCUSSION

Regeneration of shoots from leaf sheaths

The initial shoots were induced in the cutting side of leaf sheaths after 4 weeks in regeneration media. Shoots developed into clusters alternating by porous callus (Figure 2b). After 8 weeks, by the magnifying glass, it is clear to see that each bud cluster was covered by a layer, and contained shoots inside (Figure 2c and 2d). By the 12th week, the shoots grew large, turned green, and were around 3 mm in height (Figure 2e). Then, regenerated shoots were transferred to media without TDZ for plant development and root formation (Figure 2f). Those shoots started to form roots after two weeks in medium containing 3 mg/L BA and we could get in vitro ginger plants with roots growing well after about nine weeks (Figure 2g).

The shoot’s regeneration data were collected at 15th weeks in all treatments. The highest regeneration rate (68.33%) was obtained in medium containing 1 mg/LTDZ. In addition, this treatment also produced highest shoot number per sample (24.63 shoots) (Table 1). The combination between TDZ and 0.5 mg/L IBA gave the lower efficiency in both shoot regeneration and average number of shoot when compared to those of treatments without IBA (Table 1). These results were different to those of Lincy (2009). They could induce the direct regeneration of shoots from aerial stems of Jamaican ginger variety with 100% in efficiency, when using 1 mg/L TDZ and 0.5 mg/L IBA (Lincy et al., 2009). However, according to Aasim (2009), when using TDZ alone, the regeneration rate and the number of regenerated shoots were high, but the height of those shoots were limited by the inhibition effect of TDZ. The presence of IBA could increase the height of the regenerated shoot, but reduced the regeneration rate as well as the number of regenerated shoots (Aasim et al., 2010). In this study, there were the same issues, and to solve this disadvantage of TDZ, all regenerated shoots were moved to other media without TDZ to develop height of shoots and induce roots. In another report, the combination of TDZ in range of 0.1-1.0 mg/L with 2 mg/L 2,4-D were applied on leaf sheaths of 3-month-old in vitro explants of Indian ginger cv "Maran". The results indicated that it could induce embryogenic callus with efficiency in the range of 47.5-82.03% (Musfir Mehaboob et al., 2019). In current time, there are several reports on shoot regeneration of Z. officinale from different materials such as young leaves, shoot tips and sprouts from rhizome using both organogenesis and somatic embryogenesis systems. However, the protocols and efficiencies are quite different, and highly depend on genetic resource, as subspecies or varieties (Ali et al., 2016; Ibrahim et al., 2015; Lincy et al., 2009; Mehaboob et al., 2019; Sathyagowri et al., 2011; Solanki et al., 2014; Suma et al., 2008; Tran et al., 2010). This is the first report of shoot regeneration from in vitro leaf sheath (in vitro explant) of Vietnamese Z. officinale. It has potential to provide the stable source, and effective method to the application on genetic transformation in Vietnamese Z. officinale.
Table 1. Effect of TDZ and IBA on shoot regeneration from leaf sheaths

<table>
<thead>
<tr>
<th>IBA (mg/L)</th>
<th>TDZ (mg/L)</th>
<th>Shoot regeneration rate (%)</th>
<th>Average number of shoot/regenerated sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5</td>
<td>33.89 ± 2.00 c</td>
<td>14.31 ± 0.30 d</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>68.33 ± 0.96 a</td>
<td>24.63 ± 0.17 a</td>
</tr>
<tr>
<td>0</td>
<td>1.5</td>
<td>63.89 ± 1.47 a</td>
<td>20.18 ± 0.22 b</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>13.33 ± 1.67 d</td>
<td>9.65 ± 0.30 a</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>37.78 ± 3.09 bc</td>
<td>17.29 ± 0.24 c</td>
</tr>
<tr>
<td>0.5</td>
<td>1.5</td>
<td>46.11 ± 1.11 b</td>
<td>19.55 ± 0.13 b</td>
</tr>
</tbody>
</table>

Values were expressed as the mean ± SE (standard error), taking 30 samples in each treatment with three replications. All data were taken after 15 weeks after starting induction in regeneration media. With each treatment, the different letters showed the significant differences on values among treatments by the Tukey-HSD test (α = 0.05).

Figure 2. Regeneration of shoots from leaf sheaths

(a) Leaf sheaths are cut from 1-month-old in vitro explants. (b) 4 weeks after induction (WAI), small shoots are created in regeneration media. (c) 8 WAI, buds carry several small shoots in regeneration media. (d) Small shoots in closed-up view; (e) 12 WAI, shoot clusters develop in regeneration media. (f) Multi-shoots form roots in micro-propagation media. Scale bar: 1 mm. (g) in vitro ginger plants. Scale bar: 1 cm.

Effect of hygromycin on leaf sheaths

In this study, after 4 weeks in treatment of 6 mg/L hygromycin, no sample could survive. And after 6 weeks, no shoot was induced in treatment of 4 mg/L hygromycin. Consequently, this assessment was stopped and data were collected in all treatments after 6 weeks of screening. The results indicated that in the control media (without hygromycin) 66.67% of leaf sheaths formed healthy shoots. However, this value dropped down to 10%, when hygromycin concentration increased to 2 mg/L (Table 2). Moreover, results from other experiment showed that treatment of 4 mg/L hygromycin could inhibit the growth of more than 70% of 1 cm-shoots after eight weeks (detail data not shown). Therefore, 4 mg/L was chosen as the concentration of hygromycin to screen transgenic shoot regenerating in infected leaf sheaths. In other reports, hygromycin concentration used to select the transformants were 15 mg/L with maize callus (Walters et al., 1992), 20 mg/L with Arabidopsis (Ziemienowicz, 2001), and more than 50 mg/L with some grasses and monocotyledonous plants (Miki, McHugh, 2004). However, in this study, the suitable concentration is only 4 mg/L, much lower than those of mentioned reports.

Table 2. Effect of hygromycin on shoot regeneration from leaf sheaths

<table>
<thead>
<tr>
<th>Hygromycin (mg/L)</th>
<th>Rate of samples forming shoots (%)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>66.67 ± 1.67</td>
<td>Healthy shoots</td>
</tr>
<tr>
<td>2</td>
<td>10.00 ± 2.89</td>
<td>Porous calli were formed in most of samples</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>Porous calli were formed in some samples</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>All samples died after 4 weeks</td>
</tr>
</tbody>
</table>

Values were expressed as the mean ± SE (standard error), taking 20 samples in each treatment with three replications. All data were taken after 6 weeks from starting point.
Selection of transformed shoots, *Gus* expression and PCR detection

After 3 days of co-culture period, infected samples were sub-cultured in regeneration media supplemented with 4 mg/L hygromycin every two-month until forming resistant shoots. In this study, the hygromycin-resistant shoot clusters were obtained after 10 weeks of infection (Figure 3a). Nine shoot clusters were got (from more than 1000 infected leaf sheaths) and each of them were cut a small part to perform histochemical *Gus* assay. The results showed that the blue color of X-Gluc stain was observed in the base of shoot cluster, but not in the upper part (Figure 3b and 3c). All hygromycin-resistant shoot clusters were maintained in regeneration media with 4 mg/L hygromycin until forming full shoots. After that 10 weeks, the *Gus* assay was performed with four lines of grew resistant-shoots which were cut in half vertically. The blue color of X-Gluc stain was really light and only observed in the base of shoot, but not in the leaf blades (Figure 3d).

To identify the presence of transgenes in *Z. officinale* regenerating shoot lines, molecular analysis was performed by PCR method. First, to confirm the absence of *A. tumefaciens* on hygromycin-resistant shoot clusters, leaf blades were cut and shaken in 1.5 mL tubes (containing 1 mL LB) with 200 rpm at 37°C for one hour. After that, leaves were discarded and 1 mL solution was centrifuged at 3500 rpm for 10 minutes to get pellets. Then, pellets were resuspended in 100 µL LB and spread on LB plates. These plates were incubated at 28°C for 48 hours. No colony appearing on these plates indicated that *A. tumefaciens* did not remain on resistant shoot clusters. Next, *in vitro* leaf blades from each shoot clusters were collected and extracted genomic DNA. PCR analysis was carried out by using two specific primer pairs of selectable maker gene (*hpt*) and target gene (*Ca-AFP*). The specific DNA fragments were amplified, with 508 bp and 300 bp for *hpt* and *Ca-AFP*, respectively. The obtained results after electrophoresis indicated that there are four transgenic lines harboring both *hpt* and *Ca-AFP* genes, with the respective bands in lanes 3 - 6 in Figure 4a, and 4b.

In previous study, transgenic ginger was transformed with reporter gene (*gus*), or selectable marker gene (*hpt, nptII*) in order to study the genetic transformation in *Z. officinale* (Musfir Mehaboob et al., 2019; Suma et al., 2008). It is obvious that transformation in ginger has great potential. In this study, we showed initial result of transformation of a plant defensin gene (*Ca-AFP*)-target gene-into ginger leaf sheaths to create transgenic plants.

CONCLUSION

This is the first time that shoots regenerated from *in vitro* leaf sheaths of Vietnamese ginger by using TDZ. This material was applied on genetic transformation of *Z. officinale* with *Ca-AFP*, a defensin gene with antifungal


NGHIEN CƯU TÀI SINH TỪ NGUỒN MÃO IN VITRO VÀ BUỘC ĐẦU BIẾN NẠP GEN KHÁNG NẤM VÀO CÂY GỪNG (Zingiber officinale Rosc.) BẰNG VÌ KHUANH Agrobacterium
Trần Thị Ngọc Hà*, Nguyên Hữu Hồ, Nguyễn Hữu Tâm, Lê Tấn Đức, Phan Trường Lộc
Viện Sinh học Näht đổi, Viện Hành lâm Khoa học và Công nghệ Việt Nam

TÔM TÁT
Các nghiên cứu trước đây cho thấy, cây gừng (Zingiber officinale Rosc.), cũng như các loại khác trong chi Zingiber, rất khó tái sinh chỉ từ nguồn mâu in vitro. Trong nghiên cứu này, chúng tôi khảo sát môi trường tái sinh cho bẹ lá gừng (leaf sheath) in vitro. Kết quả cho thấy môi trường MS chứa 1 mg/L TDZ cho hiệu quả cao nhất với 68,33% mâu tái sinh và trung bình 24,63 chồi hình thành trên mỗi mâu tái sinh. Sau đó, kết quả này được áp dụng cho quy trình biến nạp gen ở cây gừng. Gen kháng nấm Pythium aphanidermatum cho hiệu quả cao in vitro đồng thời với gen β-glucuronidase (gus) và gen hygromycin phosphotransferase (hpt), bằng cách sử dụng vi khuẩn Agrobacterium tumefaciens. Kết quả ban đầu cho thấy, sau 10 tuần trong môi trường chọn lọc có chứa 4 mg/L hygromycin, sự biểu hiện của gen gus đã được ghi nhận ở một số chồi tái sinh. Sau 20 tuần chọn lọc trên môi trường có chứa 4 mg/L hygromycin, chúng tôi đã nhận được bốn dòng chồi tái sinh mang gen Ca-AFP và hpt thông qua phương pháp PCR. Kết quả của nghiên cứu này cho thấy tiềm năng tạo ra một giống gừng mới có thể cải thiện khả năng kháng bệnh thời điểm do nấm Pythium aphanidermatum trong tương lai.

Keywords: Agrobacterium tumefaciens, gen kháng nấm, tái sinh, Zingiber officinale Rosc.

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