Evaluation of Crown Gall Disease and Root Rot Disease in Hybrids of *Rosa* ‘PEKcougel’ and *R. multiflora* ‘Matsushima No. 3’ (*Rosa* ‘PEKcougel’と *R. multiflora* ‘Matsushima No. 3’の交雑集団における根頭がんしゅ病と根腐病抵抗性の評価)

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INTRODUCTION

Crown gall disease caused by *Agrobacterium tumefaciens* is a common disease usually occur in many fruit trees, grapevine and some ornamental crops (Bliss et al., 1999). *A. tumefaciens* is a soil borne pathogen which has a wide host range and mediates gene transfer during infection (De Cleene and De Ley, 1976). Generally, crown gall disease is not lethal for plant, however, it can cause great economic losses due to the quality degraded and yield reduction (Poncet et al., 1996). As one of the important ornamental crops around the world, Rose is suffering from crown gall disease (Zhou et al., 2000).

A. tumefaciens had a wide range of hosts, 643 plants are belonging to 331 genera and 93 families are host plants of *A. tumefaciens* (De Cleene and De Ley, 1976). Crown gall disease was considered the main reason caused great economic loses to nursery production, such as apple, grapevines, walnut, cherry, and ornamental plant such as rose (Garrett, 1987; Poncet et al., 1996; Escobar et al., 2002; Moriya et al., 2008; Filo et al., 2013). For instance, Chen et al (2007) reported that the crown gall disease seriously reduced the quantity and quality of the grape production, in some grapevine yards, 70% of grapevine showed disease incidence. For the rose production, the mortality after inoculated was 10 times higher than the control group, and the flower production reduced from 9.7 to 6.2 per plant, compared to the control (Poncet et al., 1996). Pulawska (2010) mentioned that crown gall stunted the growth of young cherry.

It has been well studied that there is a specific fragment called T-DNA in the

tumor-inducing (Ti) plasmid from *A. tumefaciens* (Tinland, 1996). On T-DNA fragment, the genes critical for crown gall disease can be divided into two groups, oncogene and opine gene (Bourras et al., 2015). Among the oncogenes, three of them are critical for cell proliferation and crown gall development, tryptophan-2-monooxygenase (*iaaM*), indole-3-acetamide hydrolase (*iaaH*) and isopentenyl transferase (*ipt*). The *iaaM* and *iaaH* encode enzymes for biosynthesis of auxin and *ipt* encode enzymes for cytokinin (Klee et al., 1984; Lichtenstein et al., 1984). The expression of oncogenes can lead to produce excess plant hormones, show as uncontrol callus growth, and *iaaM* and *ipt* were critical for this progress (Escobar et al., 2001). The expression of opine gene after gene transfer can provide a carbon/nitrogen source for *A. tumefaciens* (Coenen and Lomax, 1997). By either prevent the T-DNA transfer, or to regulate the oncogene expression in the infected plant cell, can reduce the disease severity (Narasimhulu et al., 1996; Hansen, 2000; Tan et al., 2004; Anand et al., 2007). Some characteristics of plants can also relate to tumor growth (Zhang et al., 2015).

Since the pathogen of crown gall disease was discovered, the management of this disease is generally focusing on phytosanitation (Escobar and Dandekar, 2003). Recently, biocontrol has been proved to be an effective way to control crown gall disease. It has been reported that many nonpathogenic *A.* can be used as biocontrol agents such as *A. radiobacter* K84, *A. vitis* VAR03-1, *A. vitis* ARK-1, and even genetically engineered *Agrobacterium* strain K1026 (Kawaguchi et al., 2008, 2015; McClure et al., 1998; Penyalver et al., 2000). Although these biocontrol agents have been proved to be effective against pathogens, the strain of pathogens and the plant species can significantly affect the effectiveness of the biocontrol agents (Kawaguchi et al., 2015). Moreover, some researchers focus on the plant responses to *A. tumefaciens* infection, try to breed disease resistant plants. Hereby, we would like to introduce the

management strategy from three aspects, environment, biocontrol and plant response.

1. Environment

The most important thing to control crown gall disease during agricultural production is to culture carefully. *A. tumefaciens* infection required wound, so that the bacteria can attach to the cell surface and initiate horizontal gene transformation. It had been reported that controlling the population of chewing insects by any method can be critical for disease management (Burr and Otten, 1999). Besides, during the growing season, applied copper fungicides or copper-based compounds, avoid wet and heavy soil can help to control grapevine crown gall disease (Burr et al., 1998).

2. Biocontrol

After the T-DNA transformation, the tumor-like tissue can grow without the existence of bacteria and exogenous (Braun, 1958). Therefore, to protect the plant cell from forming gall tissue caused by *A. tumefaciens* infections, the key is to eliminate the bacteria before T-DNA transfer happened. So far, many avirulent *Agrobacterium* showed their ability to control crown gall disease by antagonism. It had been reported that *A. radiobacter* strain K84 is the most widely used and successful method to control crown gall disease (Penyalver et al., 2000). The ability that K84 can control crown gall disease is basically due to the antibiotic called agrocin 84, which can terminate DNA synthesis in bacteria (New and Kerr, 1972; Kerr and Htay, 1974). When the bacteriocin sensitive pathogenic strain exposed to K84 strain, the resistant colonies developed but the pathogenic strain became avirulent (Kerr and Htay, 1974). Ryder and Jones (1991) reported that the percentage of tumor occurrence of both young and old almond seedlings reduced to 14% and 20% respectively after treated with K84 strain compare

100% to the control. Besides, K84 strain can completely inhibit the gall formation at a pathogen to nonpathogen population ratio of 1:1 or less in peach seedlings (New and Kerr, 1972). Interestingly, agrocin 84 resistant pathogenic strain *Agrobacterium* B6 and 66R even not be inhibited *in vitro*, but tumor formation was significantly reduced by strain K84 *in vivo* (Penyalver and López, 1999). However, Ellis et al. (1979) reported that after the agrocin 84 synthesis plasmid transfer conjugatively to pathogenic agrobacteria, made them insensitive to agrocin 84. By using kanamycin-resistance transposon Tn5 marked pAgK84, Farrand et al. (1985) found that the conjugal transfer not only occurred between *Agrobacterium* strains, but also *Rhizobium meliloti*, contributed immunity against agrocin 84. Ryder et al. (1987) finally confirmed that the immunity encoded region on pAgK84. Thus, *A. radiobacter* strain K1026, a genetic engineered derivative of K84, which will not transfer agrocin 84 synthesis plasmid had been created, showed the same ability to control crown gall disease like K84 (Jones and Kerr, 1989).

Furthermore, Kawaguchi and Inoue (2012) reported that *A. vitis* strain ARK-1, ARK-2 and ARK-3 can reduce tumor incidence. Interestingly, these three biocontrol agents did not produce a halo of inhibition on medium. Another nonpathogenic *A. vitis* Strain VAR03-1 can effectively control crown gall caused by tumorigenic *A. vitis*, *A. rhizogenes*, and *A. tumefaciens* (Kawaguchi et al., 2008). In addition, not only nonpathogenic *Agrobacterium*, but other microorganisms had also been reported to be functional controlling crown gall disease. For example, *Rahnella aquatilis* HX2 that isolated from soil showed great inhibition effect on the crown gall development in grapevine (Chen et al., 2007). Furthermore, Hammami et al. (2009) reported that *Bacillus subtilis* strain 14B can control *Agrobacterium* spp. by producing bacteriocin called Bac 14B. While Frikha-Gargouri et al. (2017) found that *Bacillus*

methylotrophicus strain 39b can suppress the growth of tumorigenic *A. tumefaciens*. In another experiment, *Serratia plymuthica* IC1270, *Pseudomonas fluorescens* Q8r1-96 and *Pseudomonas fluorescens* B-4117 can strongly suppress *Agrobacterium* growth by their secretion (Dandurishvili et al., 2011).

3. Plant response

The resistant mechanism of plants against *A. tumefaciens* can be divided into two parts. First is to prevent the T-DNA transfer, second is to regulate the oncogene expression in the infected plant cell (Narasimhulu et al., 1996; Hansen, 2000; Tan et al., 2004; Anand et al., 2007).

When the *A. tumefaciens* attached to plant cell, some compounds from the plant can induce the *vir*-genes expression, which is necessary for the T-DNA transfer. Stachel et al. (1985) reported that the acetosyringone (AS) and α-hydroxyacetosyringone (OH-AS) from plant wounds can induce the *vir* regulon and the formation of T-DNA intermediate molecules. Furthermore, Sheikholeslam and Weeks (1987) reported that transformation rate of *Arabidopsis thaliana* leaf explants after adding AS ranged from 55-63%, while the control was 2-3%. On the other hand, the *vir*-induced virulence protein products were induced by AS (Engström et al., 1987). Shaw et al. (1988) further proved that the *virA* and *virG* mediated chemotaxis at a low concentration of AS, and effect *vir*-induction at a higher concentration of AS. Not only in the model plant, in apple, *A. tumefaciens* transformation was significantly induced by AS and betaine phosphate (BP) (James et al., 1993). Tan et al. (2004) also found that resistance rose had fewer acetosyringone derivatives than the susceptible rose.

In addition, some proteins in plants had been proved that were critical for *vir*-genes expression. It had been reported that VirE2-interacting protein 1 (VIP1) in *Arabidopsis*

was required for VirE2 gene nuclear import (Tzfira et al., 2001). Ward et al. (2002) also reported that VIP1 deliver the T-strand to chromatin. Besides, overexpression of VIP1 can increase the efficiency of infection (Tzfira et al., 2002). Moreover, by reverse genetics, Li et al. (2005) demonstrated that VIP1 involved in transient transformation and interacted with the *Arabidopsis* H2A histone which required for tumorigenesis. Not only VIP1, but also VirE2-interacting protein 2 (VIP2) was required for *Agrobacterium*-mediated stable transformation (Anand et al., 2007).

Instead of not producing the *vir*-genes inducer, some plants chose to produce inhibitor. A heat-labile inhibitor in corn seedlings was identified as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), that 0.1 mM concentration of DIMBOA can completely inhibit *vir*-gene induction, and 0.3 mM can inhibit bacteria growth for 220 hours, while the concentration in seedlings is 1.5 mM or more (Sahi et al., 1990). Furthermore, Zhang et al. (2000) found that not only DIMBOA, but also 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA), root exudate of corn seedlings, inhibit the *vir*-gene expression, resulted in low transformation efficiency. In another experiment, (Maresh et al., 2006, 2007) reported that 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (HDMBOA), naturally occurring in corn seedlings, and 3-hydroxy-4,6-dimethoxy-3H-isobenzofuran-1-one (HDI), which is a synthetic analog of HDMBOA and DIMBOA, can inhibit the expression of *vir*-genes.

After the T-DNA transformation is succeeded, the expression of oncogenes can be controlled by the plant cell. Zhang et al. (2015) reported that the intergenic regions between oncogenes play a role as promotors. They also found that the WRKY18, WRKY40, WRKY60 and ARF5 in Arabidopsis were activator of *ipt* promotor, and the *wrky* mutants showed significantly smaller crown gall and lower *ipt* expression. Traditional way to breed crown gall resistant plants needs from large scale screening.

To obtain crown gall resistant plants more effectiveness, RNA interference (RNAi) had been applied. Since it had been reported that the oncogenes are highly conservative, Escobar et al. (2001, 2002) reported that by silencing the *ipt* and *iaaM* expression in *Arabidopsis*, tomato and English walnut (*Juglans regia* L.), the *Arabidopsis* RNAi line showed 0-1.5% tumorigenesis, while the wildtype is 97.5%, tomato RNAi line showed 0-24.2% compare to control 100%, and the walnut RNAi line also showed great resistant ability. However, the long-term prevention of gene silencing remained to be seen. Also, Viss et al., (2003) designed a transgenic apple tree which can prevent crown gall disease by silencing *ipt* and *iaaM* expression, while Galambos et al., (2013) further reported that crown gall resistance in transgenic grapevine was correlated with the expression of the silencing genes, and the resistance ability was strain-specific. Furthermore, silencing one oncogene can also enhanced disease resistance ability in some plants. In *Arabidopsis*, lower *ipt* expression led to smaller tumor size (Zhang et al., 2015). However, silencing only *iaaM* could enhance crown gall resistance in apple (Viss et al., 2003), suggested that the key oncogenes could be different among different plants.

Purpose

Crown gall disease caused by *A. tumefaciens* impedes rose production in Japan (Zhou et al., 2000). It had been reported previously that *R.*'PEKcougel' showed resistant ability against crown gall disease by blocking the attachment between bacteria and the wound surface with exudates, while commonly used rootstock *R. multiflora* showed great variability against crown gall disease depending on different varieties, and *R. multiflora* 'Matsushima No. 3' showed no such resistant mechanism (Reynders-Aloisi et al., 1996; Zhou et al., 2000; Tan et al., 2004; Li et al., 2008). In a preliminary

experiment, we found that *R. multiflora* ‘Matsushima No. 3’ (tetraploid) did not form typical tumor tissue. Therefore, we used *R.* ‘PEKcougel’ hybridized with *R. multiflora* ‘Matsushima No. 3’ (tetraploid) to breed the rootstock that can help to manage the crown gall disease. Traditional screening required a large amount of plant and cost a lot of time. In this experiment, our goal was to find a time-saving screening method for crown gall resistance in rose. By needle prick testing, opine detection, and measuring the expression of oncogenes, the resistant ability of these progenies had been evaluated.

Needle Prick Experiment

MATERIALS AND METHODS

Plant materials and growth conditions

Rosa 'PEKcougel', tetraploid of *R. multiflora* 'Matsushima No. 3' and their BC_1 generation were used for crown gall resistance screening (Table 1). Needle prick inoculation was conducted by using rooted cuttings. The stem was cut into small segments depending on the length of the internode. Each segment contained one lateral bud. The segments were placed in water saturation rock wool and sealed with a plastic bag. The bag was placed under 25ºC and with 24 h light (160 μmol·m^{-2}·s^{-1}) for 4 weeks for root forming. Afterward, the individual with root will be transferred to a germination tray full of germination soil BM2 (Berger, Canada) under 25ºC, 45-60% humidity and 24 h light (160 μmol·m^{-2}·s^{-1}) for acclimatization for 1-2 months before inoculation. After inoculation, the growth condition remained the same. The symptom was observed 5 weeks after inoculation.

Pathogen preparation and inoculation

A. tumefaciens 'GOU1' was used as the pathogen (Zhou et al., 2000). The strain was first cultured on solid YEB medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 493 mg/L $MgSO_4 \cdot 7H_2O$, and 15 g/L agar, pH 7.2) under darkness at 25ºC for 48 h. Before inoculation, the strain was transferred to YEB broth medium and cultured under darkness at 25ºC and 120 rpm for 24 h. Finally, the inoculum was adjusted to approx. 2×10^7 CFU/ml by optical density (OD_{600}). As for needle prick inoculation to rooted cuttings, the new shoot that grew from the lateral bud was used to conduct inoculation by needle prick inoculation method (Tolba and Zaki 2011). Depending on the shoot length, at least five places were pricked, and at least 3

cuttings were used. dH_2O was used for mock inoculation.

RESULTS

Crown gall resistance evaluation by needle prick

The appearance and the fold change of the inoculated site were shown in Figure 1 and 2. Susceptible variety *R*. 'Dukat' formed a bigger tumor size than other tested samples (Fig. 1, 2). Among the tested progenies, *R*. 'PEKcougel' showed the biggest fold change of inoculated site, significantly greater than other tested individuals (Fig. 2). The fold change of P1-4, M6-2 and M6-7 were 2.34, 1.81 and 1.99, respectively, lower than *R*. 'PEKcougel' but not significantly. The fold change of P6-4, M1-9, M1-12, M1-15, M1-23, M1-30, M1-45, M6-4 and tetraploid of *R*. *multiflora* 'Matsushima No. 3' were significantly lower than *R*. 'PEKcougel'. However, because of the low rooting percentage, it is difficult to conduct the needle prick test for the other progenies. Thus, we tried inoculation of *A. tumefaciens* using stem segment culture, opine assay and oncogene expression analysis to evaluate the resistance ability against crown gall disease.

Culture Method Establishment for Stem Segment

As a rapid detection method, opine assay will be possible to replace the traditional needle prick testing. Thus, to minimize the effects of browning during tissue culture, we tested some progenies to establish an appropriate method for stem segment culture.

MATERIALS AND METHODS

Plant materials and growth conditions

Except the plant materials were listed in Table 1, we added *R.* 'Dukat' in this experiment. Young shoot was harvested and washed using neutral detergent, then washed by tap water for 20 min. Afterward, the shoot was rinsed by 70% ethanol for 30 s, then stem surface sterilization for 20 min by 1% (active chlorine, v/v) sodium hypochlorite containing 0.01% (v/v) tween-20. The shoot was divided into three parts, internode from the leave were expending, internode from the area between expending and expanded, and the internode from expanded area. Each internode was cut into 5 mm length separately. After cutting the shoot, the stem segments were soaking in sterilize dH_2O for 5 min following inoculation. After inoculation, the stem segments were grown on hormone free MS medium (30 g/L sucrose, 2 g/L gellan gum, pH 5.7) for 72 h in growth chamber with 16 h light (120 μmol/m^2/s) at 25°C. Subsequently, the stem segments were shacked and washed with cefotaxime sodium solution for 1 minute at the concentration of 100 μg/ml, 200 μg/ml and 500 μg/ml, respectively. after washing with antibiotic, the stem segments were grown on three kinds of MS medium (hormone free; BAP 1mg/L and NAA 0.1 mg/L; BAP 1 mg/L and NAA 0.3 mg/L) contained 100 μg/ml cefotaxime sodium in the growth condition same as previously.

Pathogen preparation and inoculation

Pathogen preparation is as same as described above. The stem segments were soaked into bacterial solution for 5 min, replicant for three times.

Browning observation, logistic regression analysis and opine assay

The browning situation of each stem segment was observed a total of four weeks, record once a week. The browning index was calculated by the formula below. The rating scale used was as follows: stem was green = 0, turning brown = 1, and browned = 2. Furthermore, we used the browning rating scale that recorded each week and callus condition (negative = 0, positive = 1) to conduct logistical regression analysis to find the appropriate time for tissue culture.

Formula to calculate browning rate：

$$\frac{Green\ segment\ number \times 0 + Browning\ segment\ number \times 1 + Browned\ segment\ number \times 2}{Totle\ number \times 2} \times 100\ (\%)$$

The callus tissue from 5 mm length stem segments was carefully isolated and squashed in a 1.5 ml tube 4 weeks after inoculation. 3 μl supernatant was spotted on the chromatography paper (GE Healthcare Life Science) for electrophoresis (myPower II 500 AE-8155, ATTA) with the buffer made from formic acid, acetic acid and dH_2O (1:3:10, v/v/v) at 250 V for 20 min. Then, the paper was dried immediately and sprayed with dyeing solution (9,10-phenanthrenequinone solution 0.2 mg/ml in 99.5% ethanol) mixed with sodium hydroxide solution (0.1 g/ml in 60% ethanol) as 1:1 (v/v). Final observation by ultraviolet rays (312-360 nm) after the paper was dried.

RESULTS

At the very beginning, we tested if the timing that stem segments turn browned may influence the opine detection rate. We first screened the opine from the stem segments that turned browned in the 2nd week, the 3rd week and the 4th week. To find

out if the stem segment condition may influence the opine detection rate, the opine from green stem segments and browned segments had been observed. The results showed that the opine detection rate from green stem segments was higher in most samples. In some samples, the opine detection was 0% from the browning samples (Fig. 3). These results indicated that the browning progress during tissue culture had a great influence on opine detection. Thus, inhibiting the browning process will be important for opine assay.

It was obvious that the stem will turn browned as the culturing time continued. To further investigate the relationship between callus formation rate and the stem segments condition, the callus formation rate from green and browned stem segments were recorded. The results showed that when there was a callus tissue, more than 82% of the stem segments were green, 16% were during the browning process and only 2% were browned. On the contrary, if there were no callus tissue, 80% of the stem segments were browned, 19% were during the browning process, and only 1% were green (Fig. 4). To find the most appropriate time to culture the stem segments, we used logistic analysis to investigate the relationship between callus formation and the browning index (Table 2). The results showed that the stem condition in the 2^{nd} week and the 4^{th} week are highly related to callus formation, respectively. If the stem segments were browned on 14-days, then the callus formation rate had a significantly negative relationship with stem condition. However, if the stem segments were not browned in the 4^{th} week, the callus formation rate had a significantly positive relationship with stem condition.

To find the most suitable part from the shoot for tissue culture, we tested the different parts from the young shoot. We divided the shoot into three parts, internode from the leave were expanding, internode from the area between expanding and expanded, and the internode from expanded area. The results showed that the internode

from the expanding area turned browning fastest, finally over 90% browning rate. The browning rate of the internode between expending and expanded, and internode from expanded showed similar results between P and M, both browning rate from different internode of these two varieties showed much lower than the internode from the leave expending as the time increased (Fig. 5). Moreover, we investigated if the hormone medium can inhibit or slow down the browning process. Figure 6 showed that after hormone application, the browning process of the stem segments was slower than hormone free medium, especially in the 4th week.

The longer the cultivation time, the larger the tumor tissue, which will increase the sensitivity of opine assay. As time increased, the browning rate continuously increased, especially in the 4th week (Fig. 5). Besides, logistical regression results indicated the callus formation rate correlated with the browning rate in the 2nd week and 4th week, but not the 1st week or the 3rd week. Combining all together, in this experiment, the most appropriate culture time for stem segments was 3 weeks. Furthermore, we decided to use hormone free medium because the browning rate of stem on hormone medium showed a great difference only in the 4th week.

Opine Assay and Oncogene Expression Analysis

MATERIALS AND METHODS

Plant materials and growth conditions

Depending on previous results, the internode between leaf expending and expended, and only hormone free MS medium had been used. The rest condition remained the same as previous.

Pathogen preparation and inoculation

Pathogen preparation is as same as described above except the callus tissue was carefully isolated and squashed in a 1.5 ml tube 3 weeks after inoculation.

opine assay

Opine assay is as same as described above.

RNA extraction and gene expression analysis

The total RNA from 2 mm stem segments was extracted 1 week after inoculation by using the RNAs-ici!-R kit (Rizo, Japan) following the manufacturer's protocol. The genomic DNA in the extracted total RNA had removed by DNaseI (Nippon gene, Japan) for 25 min at 37ºC. DNaseI was removed by phenol/chloroform extraction method. cDNA synthesis was performed by using the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara-bio, Japan). Real-time quantitative reverse transcription PCR (qRT-PCR) was performed by using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara-bio, Japan). PCR was performed as follows: an initial denaturation of 30 s at 95°C, followed by 40 cycles at 95°C for 5s and 60.5°C for 45 s. Afterward, the dissociation step is initial at 95°C for 15s, keep 60.5°C for 30 s, and keep 15s at 95°C. The primers used in this experiment are shown in table 3.

RESULTS

Crown gall resistance evaluation using stem segment culture.

The callus formation rate of *R.* 'PEKcougel' was 43.6% while tetraploid of *R. multiflora* 'Matsushima No. 3' formed no callus tissue 3 weeks after inoculation (Table 4). Among all tested progenies, the M1 line and M6 line showed a lower callus formation rate than the P1 line and P6 line (Table 4). Furthermore, six individuals (M1-5, M1-23, M1-25 M1-30, M1-33 and M6-2) formed no callus tissue (Table 4), and all these individuals are the backcross line of tetraploid of *R. multiflora* 'Matsushima No. 3' with F_1 generation.

Among the samples which formed callus, three progenies (M1-12, M1-15 and M6-10) showed no opine detection rate (Table 4). For the tested samples which formed no callus, the further screening will be necessary. Therefore, M1-5, M1-23, M1-25, M1-30, M1-33 and M6-2 were chosen for the next oncogenes expression analysis. Moreover, individuals with the callus formation rate lower than 20% or low opine detection rate also had been chosen for gene expression analysis.

Primer specificity testing

We first tested the house-keeping gene candidates on tetraploid of *R. multiflora* 'Matsushima No. 3' and *R.* 'PEKcougel'. Figure 8 showed that all primers showed good specificity on both tetraploid of *R. multiflora* 'Matsushima No. 3' and *R.* 'PEKcougel', and the length of amplicon showed the same on different replicants. Furthermore, we also tested the *ipt* and *iaaM* primer specificity, figure 8 showed that after the plant was infected, even though a primer dimer occurred, only one band occurred around the expected amplicon, and nothing was observed from the mocking samples. Afterward, we sequenced the amplicons and compared the sequence to the database, *ipt* and *iaaM* showed 98% and 96% identities, and the *UBI4*, *UBI6* and *ACT4* of 'Matsushima No. 3'

and ‘PEKcougel’ also showed 97%-98%, 94%-98% and both 100% identities, respectively (Table 5). Thus, it is possible to use these primers to analyze oncogene expression. For the gene expression analysis, we used *ACT4* as housekeeping gene because of the 100% identities.

Oncogene expression analysis

For the expression of *ipt*, *R.* ‘PEKcougel’ showed significantly lower expression compare to P1-4 and M6-7, approximately 4 times and 3 times lower, respectively (Fig. 9 A). Combining with the needle prick results, it is obvious that these three individuals with the highest *ipt* expression level showed the greatest fold change of the inoculated site (Fig. 9A). The rest individuals showed significantly lower *ipt* expression level than *R.* ‘PEKcougel’ except M1-23 (Fig. 9A). For *iaaM*, the variation is not as great as *ipt*. Among all tested samples, P, P1-4, P1-9, M1-45 and M6-7 showed similar expression level. The rest individuals showed significantly lower expression level compared to *R.* ‘PEKcougel’ (Fig. 9B). Most of the individuals showed similar expression characteristics, two gene expressions showed positively correlated. Except for M1-45, the expression level of rest individuals was related to fold change of inoculated site. Furthermore, the relationship between oncogene expression and tumor size or opine detected rate was investigated. Results showed that both *ipt* and *iaaM* gene showed a positive correlation with the fold change of inoculated site, and *ipt* had a significant relationship with tumor size within progenies (Fig. 10). On the other hand, opine detected rate had no relationship with oncogene expression (Fig. 11).

DISCUSSION

In this study, we first screened the crown gall disease resistance ability by needle prick. The results showed that tetraploid of *R. multiflora* ‘Matsushima No. 3’ had

significantly lower fold change of inoculated cite than *R.* 'PEKcougel', and the fold change of backcross progenies were between them (Fig. 2). Interestingly, most of the backcross with tetraploid of *R. multiflora* 'Matsushima No. 3' formed no tumor tissue, suggested the resistant mechanism of tetraploid of *R. multiflora* 'Matsushima No. 3' is different from *R.* 'PEKcougel'. Unfortunately, needle prick required a large number of plant material and takes a long time, which is limited by the root forming percentage. Thus, we tried to inoculate to tissue culture and conduct opine assay to solve the problem of needle prick test.

Considering the needle prick test needs a lot of samples and time, a time-saving method will be necessary. After the T-DNA was inserted into the plant genome through Agrobacterium infection, the transformed plant cell will produce opine by the exist of opine-related genes as a substrate for *Agrobacterium* (Pedersen et al., 1983; Yang et al., 1987). Opine had been proved to be an effective method to distinguish tumor tissue (Escobar and Dandekar, 2003). Considering the opine production needs living cell, to reduce the browning during tissue culture, we established the most appropriate culture method by material selection, adjusting culture time and medium selection. Results showed that the opine detection rate from browned stem segments was lower than green stem segments, indicated the browning process can influence the opine production (Fig. 3). However, even under the most appropriate culture conditions, there were still almost one third of tested samples formed no callus tissue, and some samples showed a quite low callus formation rate or formed small callus tissue, all these limited the application of opine assay (Table 4). Thus, new method does not require callus formation will be necessary.

Currently, several mechanisms of plant resistance against *A. tumefaciens* were reported, including lack of *vir*-inducing factors (e.g. acetosyringone derivatives) and T-

DNA integration factors, plant contain antibiotic compound, as well as suppress oncogene expression in transformed cell (Sahi et al., 1990; Narasimhulu et al., 1996; Tan et al., 2004; Zhang et al., 2015; Licausi et al., 2019). For instance, silencing *ipt* and *iaaM* in Arabidopsis and tomato can enhance the crown gall disease resistance (Escobar et al., 2001, 2002; Lee et al., 2003; Zhang et al., 2015). Thus, the expression level of oncogenes (e.g. *ipt* and *iaaM*) could be used as important indexes to evaluate the resistance ability against crown gall disease in rose. In this study, we found that the individuals which showed both higher expression of *ipt* and *iaaM* had greater fold change of inoculated site than others (Fig. 2, 9). These results indicated the complexity of crown gall disease resistance mechanisms (Escobar and Dandekar 2003; Bourras et al., 2015; Kim and Park 2019). Furthermore, within the BC_1 backcross lines and their parents, the expression of both *ipt* and *iaaM* showed a positive correlation with the fold change of inoculated site, while *ipt* had significantly positive correlation (Fig. 10). The expression of *iaaM* that had no significant relationship with the fold change of inoculated site may due to insufficient sample size (Kim and Park 2019). Taken all, our results suggested that both *ipt* and *iaaM* genes are important indexes for tumor formation and development. On the other hand, opine detected rate and oncogene expression did not show any relationship. This could be explained by the reason that oncogenes were related to tumor formation and development process, while opines produced by plant only related to activate the quorum sensing of *A. tumefaciens* to further promote virulence as a nutrient source and a signal (Escobar and Dandekar 2003; Subramoni et al. 2014). In our study, some individuals showed high opine detected rate with low oncogene expression, while some individuals had high oncogene expression but with low opine detected rate (Fig. 11). This result was consistent with previous findings (Escobar and Dandekar 2003; Subramoni et al. 2014). Taken all, these results

suggested that oncogene expression analysis could be used to evaluate the progression of tumor formation and development, while opine assay could only be used to evaluate whether the plant was infected.

Besides, M1-45 showed high *iaaM* expression while the needle prick result showed resistance ability, and M6-2 showed relatively great fold change of inoculated site with low oncogene expression. The reason for this result may due to the growth promoting effect of cytokinin and auxin after infected that varies dependent on plant and tissue type (Beneddra et al., 1996; Gohlke and Deeken, 2014). Moreover, the number and types of oncogenes that need to be silenced to gain disease resistance also vary from plant species (Escobar et al., 2001, 2002; Lee et al., 2003; Viss et al., 2003; Zhang et al., 2015).

Overall, within hybrid progenies, the crown gall resistance ability of P6-4, M1-9, M1-12, M1-15, M1-23, M1-30, M1-45 and M6-4 were greater than *R*. ‘PEKcougel. Those individuals can be used to further study their resistance mechanisms and their ability to be used as rootstock. Moreover, our results showed that the oncogene expression can be used to evaluate disease resistance within rose progenies.

Table 1. Plant materials used to evaluate crown gall disease resistance and their abbreviation.

Name	Generation	Seed donor	Pollen donor	Obtained Number*
F_1 No. x	F_1	*R.* 'PEKcougel'	Tetraploid of *R. multiflora* 'Matsushima No. 3'	3
P1-x	BC_1	*R.* 'PEKcougel'	F_1 No. 1	11
P6-x	BC_1	*R.* 'PEKcougel'	F_1 No. 6	4
M1-x	BC_1	Tetraploid of *R. multiflora* 'Matsushima No. 3'	F_1 No. 1	36
M6-x	BC_1	Tetraploid of *R. multiflora* 'Matsushima No. 3'	F_1 No. 6	19

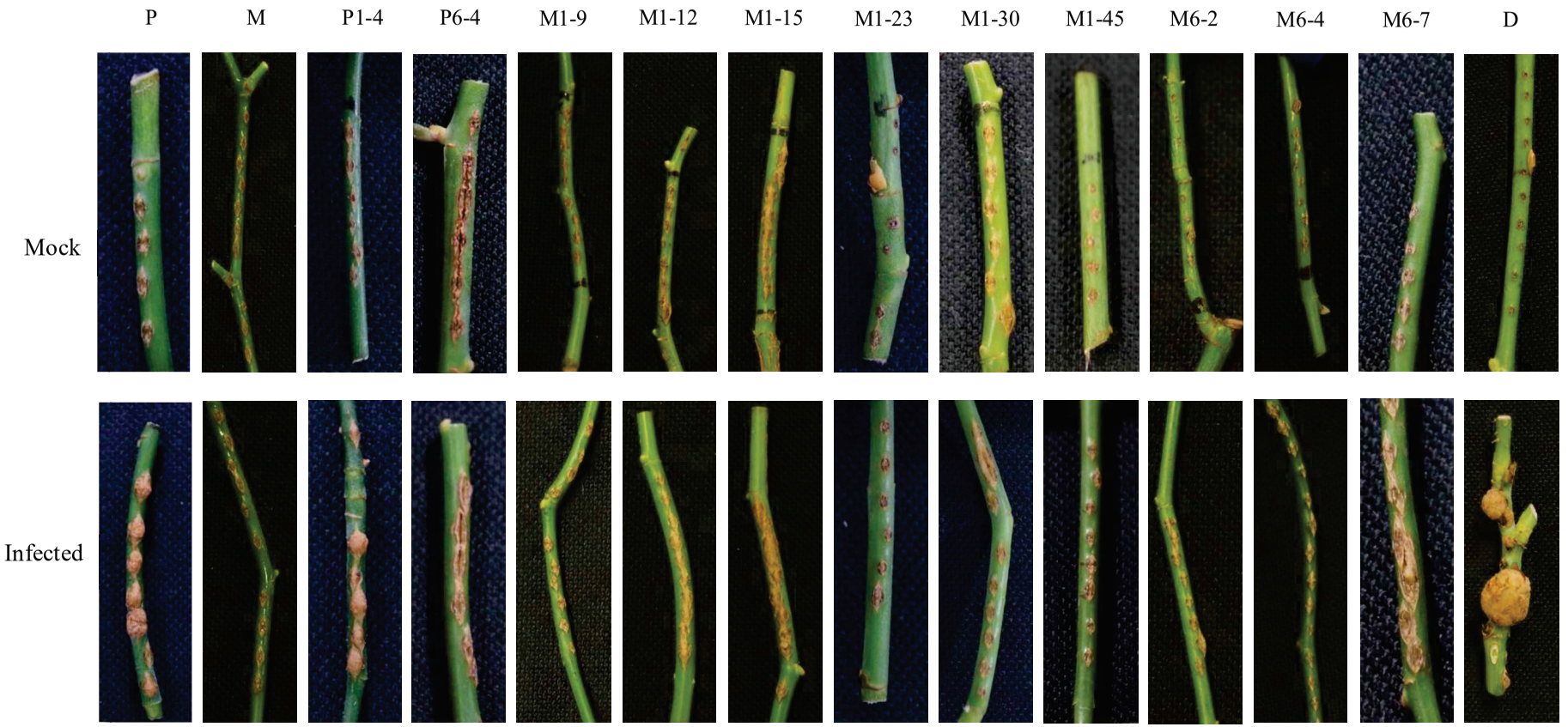

Figure 1. Inoculated site *of A. tumefaciens* 5 weeks after inoculation by needle prick. "P" represented *R.* 'PEKcougel' and "M" represented tetraploid of *R. multiflora* 'Matsushima No. 3'. "D" represented *R.* 'Dukat'.

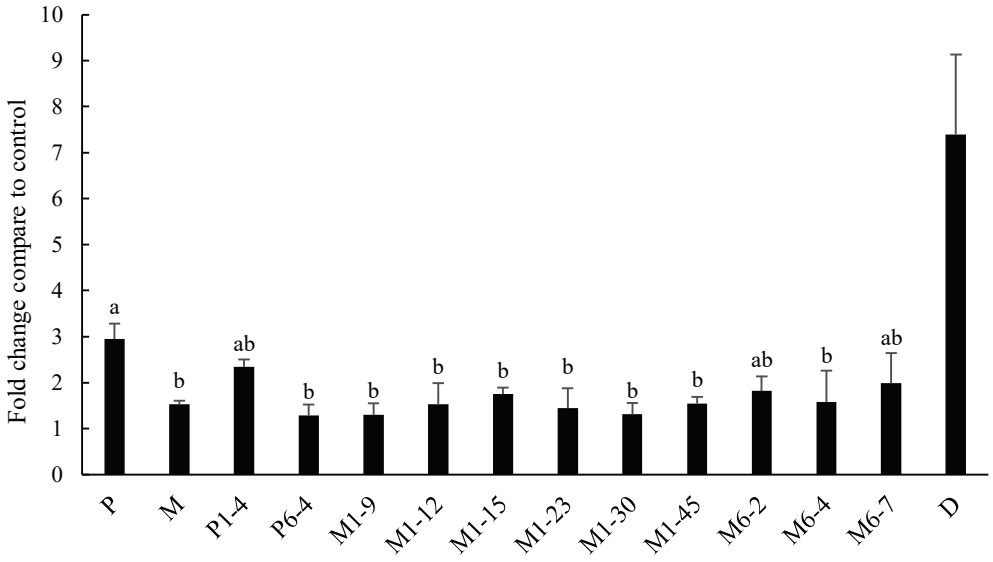

Figure 2. Fold change of inoculated site compared to mock in needle prick test. Different letters indicate significant differences at $P < 0.05$ level by using Tukey's HSD. Bars show mean values (±SD) of three independent experiments. “P” indicates *R.* ‘PEKcougel’, “M” indicates tetraploid of *R. multiflora* ‘Matsushima No. 3’ and “D” indicates *R.* ‘Dukat’.

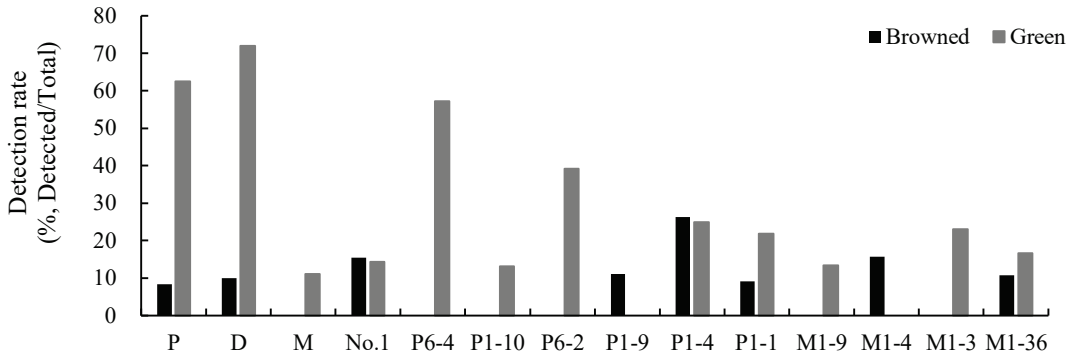

Figure 3. Opine detection rate from the stem segments comparison of browning samples and greening samples after 4 weeks subculture. “P” indicates *R.* ‘PEKcougel’, “D” indicates *R.* ‘Dukat’, and “M” indicates tetraploid of *R. multiflora* ‘Matsushima No. 3’.

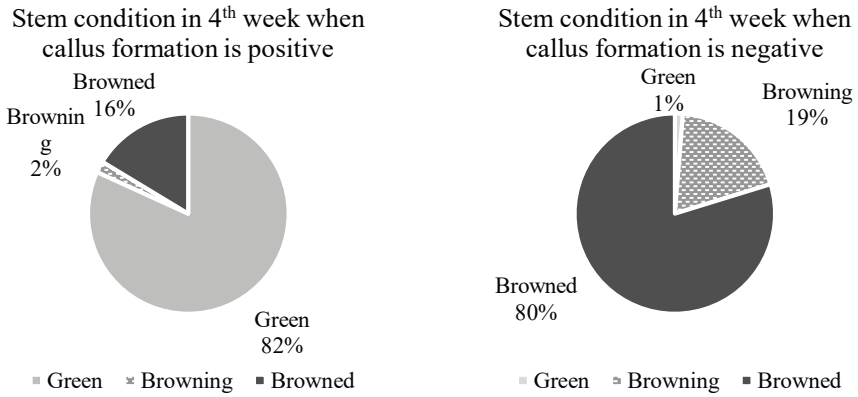

Figure 4. Comparison of stem condition in 4th week base on callus formation.

Table 2. The relationship between stem condition every week and callus formation by using logistic regression.

Variables	Regression coefficients	Standard error	P	Odds ratio	95% Confidence interval
2nd week	/	/	0.04	/	/
2nd week(1)	-1.67	0.99	0.09	0.19	0.03~1.31
2nd week(2)	-2.66	1.15	0.02	0.7	0.01~0.66
4th week	/	/	<0.01	/	/
4th week(1)	6.42	1.34	<0.01	611.96	44.16~8480.68
4th week(2)	0.24	1.28	0.85	1.27	0.1~15.63
Constant	-0.94	0.45	0.03	0.39	/

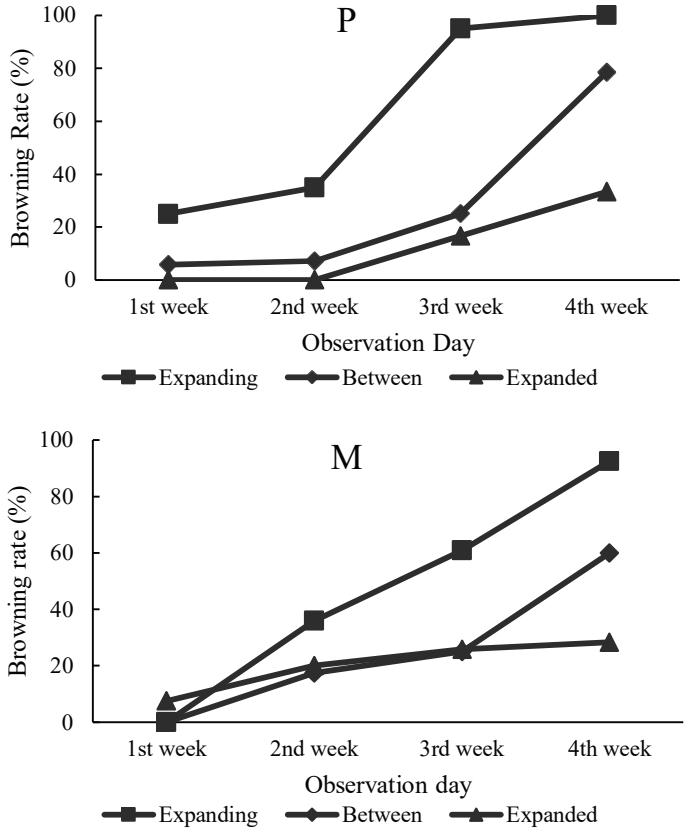

Figure 5. Browning rate by using different internode from shoot. P: The stem from *R.* 'PEKcougel'; M: The stem from tetraploid of *R. multiflora* 'Matsushima No. 3'.

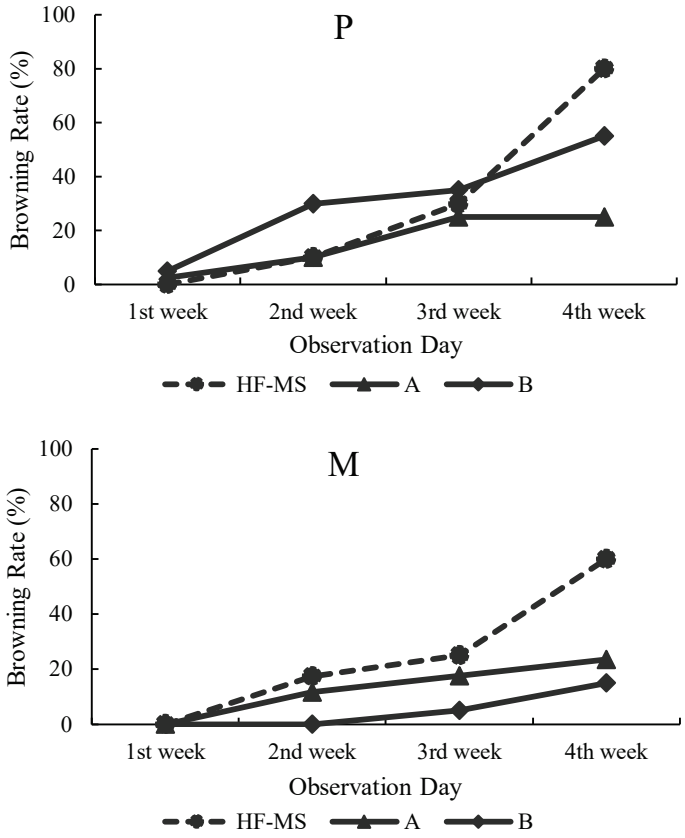

Figure 6. Browning rate by using different medium. HF-MS: hormone free medium; A: BAP 1 mg/L, NAA 0.1 mg/L; B: BAP 1 mg/L, NAA 0.3 mg/L. P: The stem from *R.* 'PEKcougel'; M: The stem from tetraploid of *R. multiflora* 'Matsushima No. 3'.

Table 3. Primer information.

Name	Primer sequence		Expected length
	Forward	Reverse	
ACT4	AAATTACTGCCTTGGCCCCT	TTTGGCGATCCACATCTGCT	131bp
UBI4	CAAAGGAAATGCTTCACAAACTGC	TCCAACATTCCAAGTCCTAATCCA	90bp
UBI6	ACATTGATTGTATCCGAAGCCA	TCCGACTTCAACCTTTCCGC	143bp
ipt	AGCCGTCTATACCTTGATGATCG	CATGCACTTGAGCAACGAGATAG	150bp
iaaM	CGCCTGTTGCTATTACTCAAGC	TCTATCGCTGAAGAGAAGGACTC	112bp

Table 4. Callus formation and opine detection 3 weeks after stem segment inoculation.

Variety or line[z]	Total sample number	Callus number	Opine detected number	Callus formation rate (%)	Opine detected rate (%)
P	39	17	7	43.6	41.2
M	20	0	-	0	-
F_1 No. 1	79	20	6	25.3	30.0
F_1 No. 6	16	3	2	18.8	66.7
P1-1	40	15	14	37.5	93.3
P1-3	65	25	12	38.5	48.0
P1-4	43	21	11	48.8	52.4
P1-9	55	16	5	29.1	31.3
P1-10	24	13	3	54.2	23.1
P6-4	46	15	6	32.6	40.0
M1-3	40	3	3	7.5	100
M1-5	39	0	-	0	-
M1-7	45	3	2	6.7	66.7
M1-9	51	1	1	2.0	-*
M1-12	137	11	0	8.0	0
M1-15	46	9	0	19.6	0
M1-23	30	0	-	0	-
M1-25	22	0	-	0	-
M1-27	43	1	0	2.3	-*
M1-30	46	0	-	0	-
M1-33	35	0	-	0	-
M1-36	47	9	8	19.2	88.9
M1-38	44	10	7	22.7	70.0
M1-45	45	3	1	6.7	33.3
M1-46	48	1	0	2.1	-*
M6-2	25	0	-	0	-
M6-4	31	4	3	12.9	75.0
M6-7	68	21	11	30.9	52.4
M6-10	53	7	0	13.2	0
D	38	26	17	68.4	65.4

[1]Callus formation rate (%) = Callus number / Total sample number × 100

[2]Opine detected rate (%) = Opine detected number / Callus number × 100

"P" represented resistant variety *R.* 'PEKcougel', "M" represented tetraploid of *R. multiflora* 'Matsushima No. 3' and "D" represented susceptible variety *R.* 'Dukat'.

*) Opine detected rate was not calculated when the callus number is less than three.

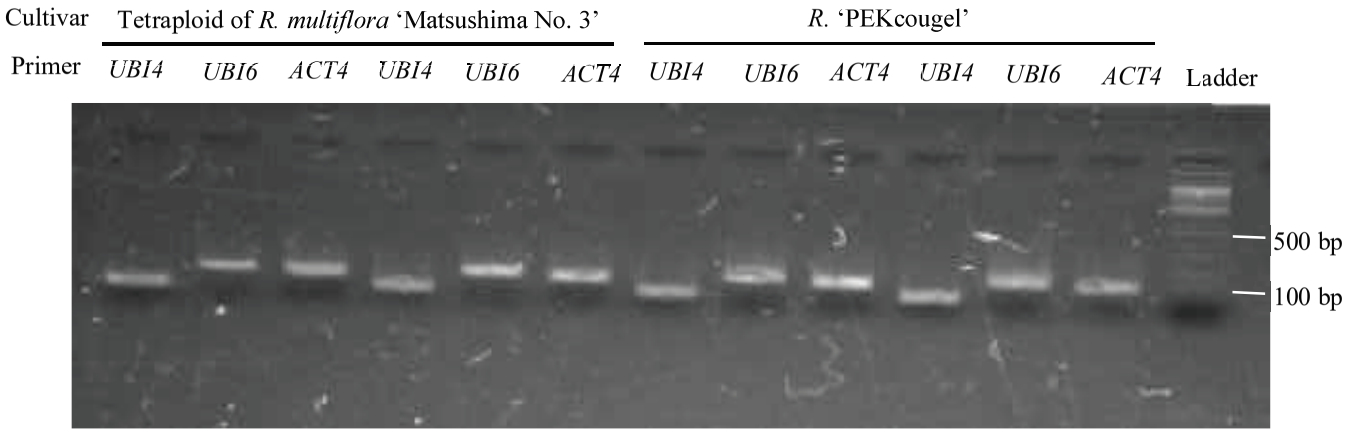

Figure 7. Electrophoresis of candidates of house-keeping genes.

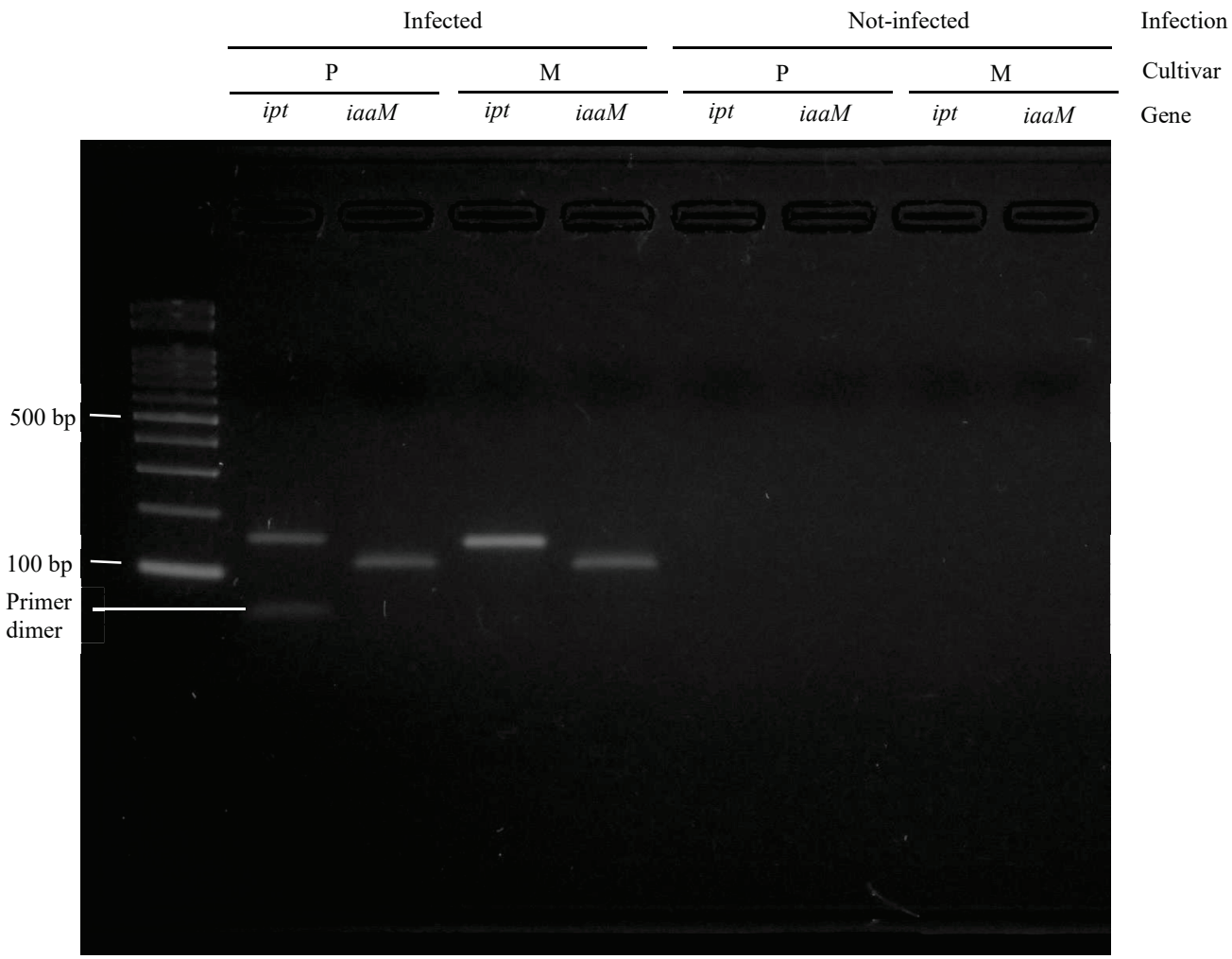

Figure 8. Electrophoresis of *ipt* and *iaaM* on inoculated and mocking samples. “P” indicates *R.* ‘PEKcougel’, “M” indicates tetraploid of *R. multiflora* ‘Matsushima No. 3’.

Table 5. Gene homology comparison by using sequenced data.

Name	E value [z]	Identities	Accession number	Definition	Organism
ipt	9.00E-19	98%	AE007871.2	*ipt*	*Agrobacterium tumefaciens* str. C58 plasmid Ti
iaaM	7.00E-25	96%	AE007871.2	*iaaM* (*tms1*)	*Agrobacterium tumefaciens* str. C58 plasmid Ti
UBI4	M: 1E-16 P: 3E-14	M: 97% P: 98%	KC514917.1	Ubiquitin family protein 4 (*UBI4*)	*Rosa hybrida* 'Samantha'
UBI6	M: 2E-47 P: 8E-44	M: 94% P: 98%	KC514922.1	Ubiquitin family protein 6 (*UBI6*)	*Rosa hybrida* 'Samantha'
ACT4	M: 3E-37 P: 4E-36	M: 100% P: 100%	KC514920.1	Actin-4 (*ACT4*)	*Rosa hybrida* 'Samantha'

[Z]) "M" represented tetraploid of *R. multiflora* 'Matsushima No. 3', "P" represented *R.* 'PEKcougel'.

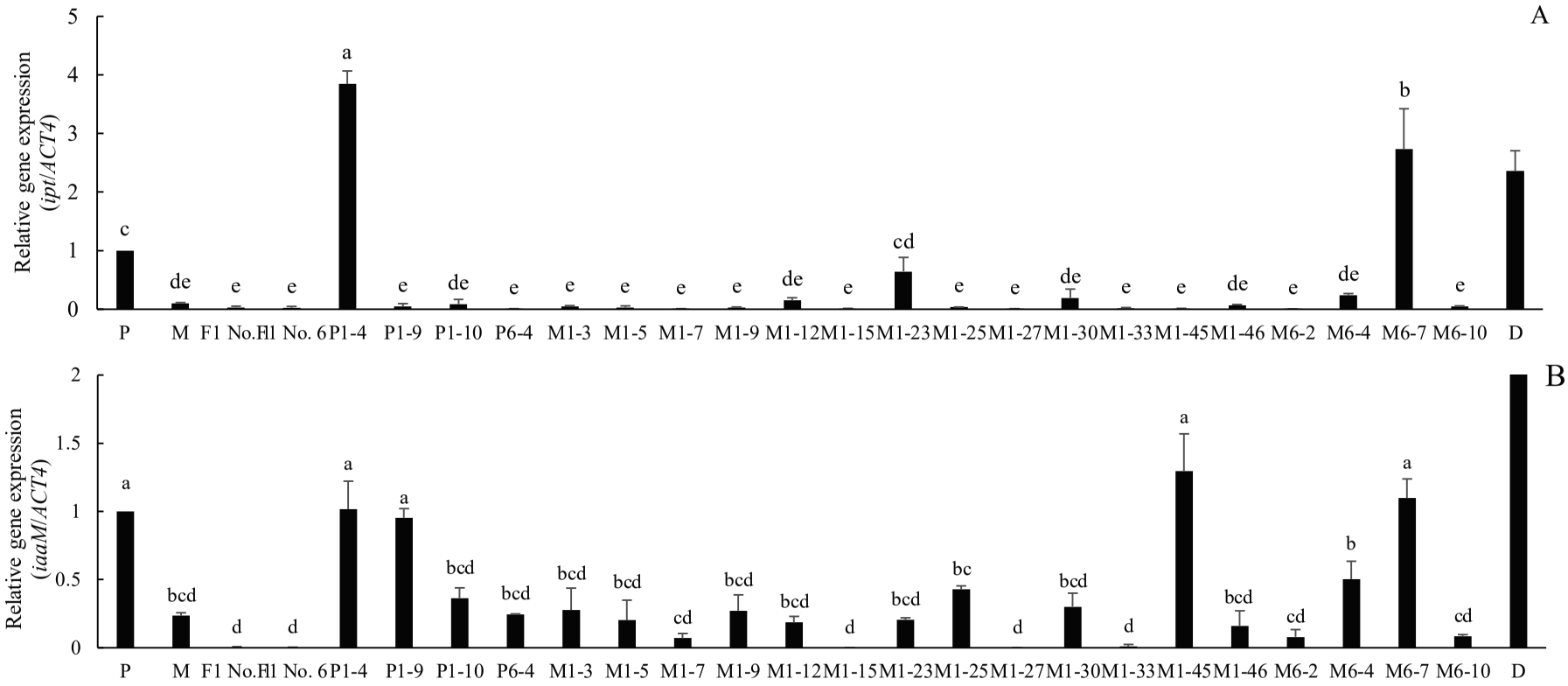

Figure 9. Expression level of two oncogenes. A: expression level of *ipt*, B: expression level of *iaaM*. Expression level was normalized by *ACT4* expression level and calibrated by P. Different letters indicate significant differences at $P < 0.05$ level by using Tukey's HSD. Bars show mean values (±SD) of three independent experiments. "P" indicates *R.* 'PEKcougel', "M" indicates tetraploid of *R. multiflora* 'Matsushima No. 3' and "D" indicates *R.* 'Dukat'.

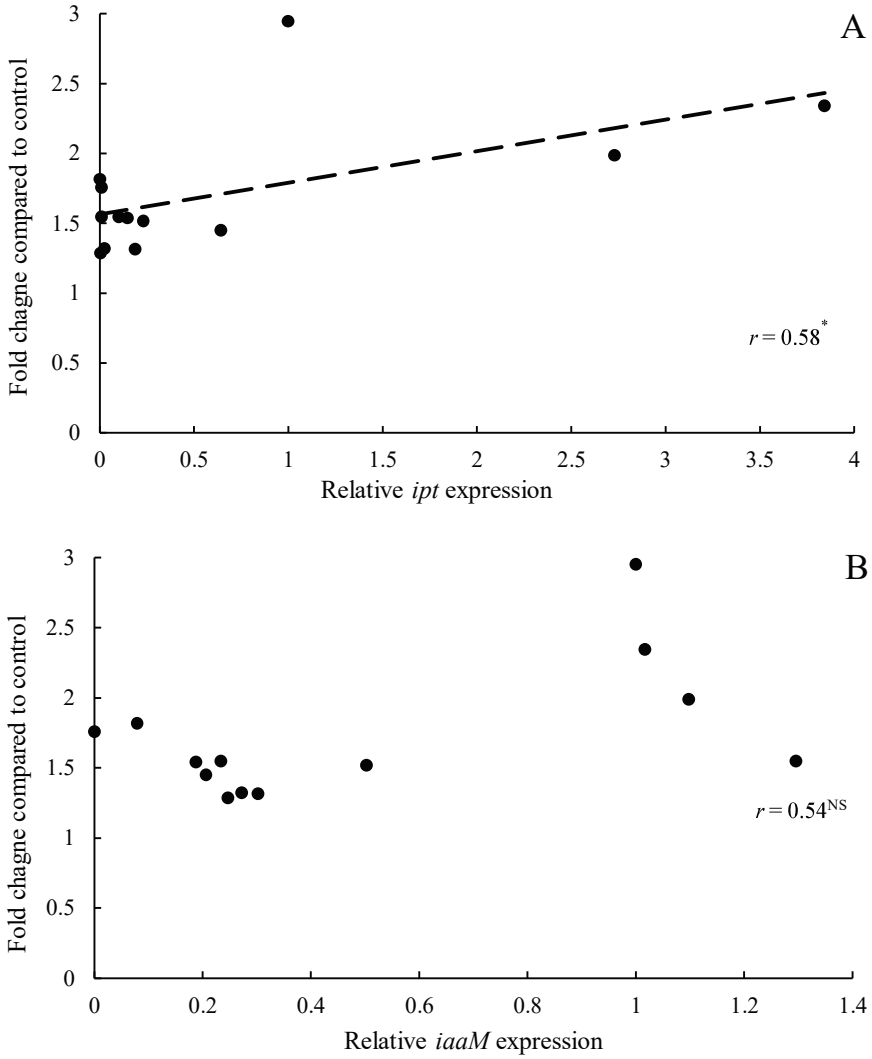

Figure 10. Relationship between fold change of inoculated site and gene expression. Relationship between fold change of inoculated site and *ipt* expression (A). Relationship between fold change of inoculated site and *iaaM* expression (B). $P < 0.05$ by using Student's *t*-test. "NS" represented nonsignificant.

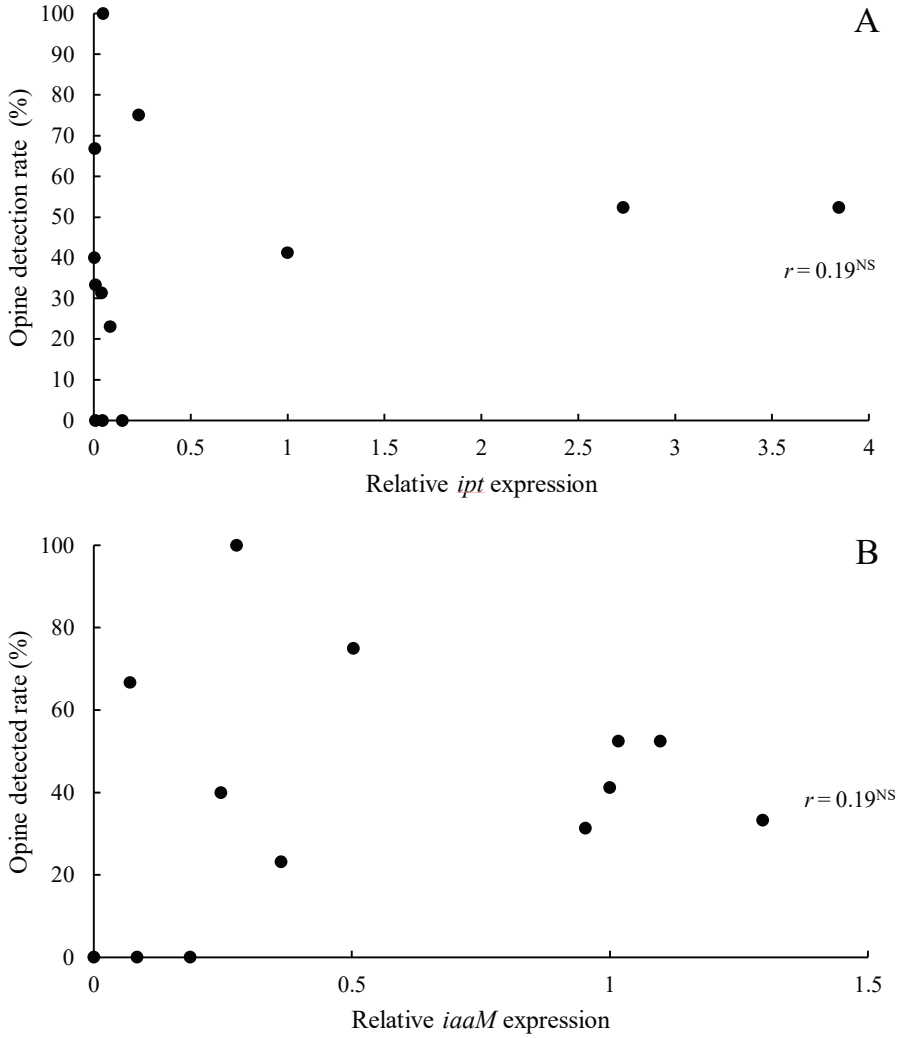

Figure 11. Relationship between opine detected rate and gene expression. Relationship between opine detected rate and *ipt* expression (A). Relationship between opine detected rate and *iaaM* expression (B). $P < 0.05$ by using Student's *t*-test. "NS" represented nonsignificant.

II. Evaluation of Root Rot Disease in Hybrids of *Rosa* 'PEKcougel' and *R. multiflora* 'Matsushima No. 3'

INTRODUCTION

Soil-borne pathogens such as *Pythium* spp. is basically difficult to control due to its highly contagious, wide range of host, and longevity, that will increase the cost of fungicide application. For instance, *Pythium* spp. can infect not only food crops (e.g. rice, wheat, and maize), but also vegetables and ornamental plants (e.g. cucumbers, sweet peppers, potatoes, tomatoes, peanut, and roses) (Johnstone et al., 2005; Li et al., 2007a; Al-Mawaali et al., 2012; Okubara et al., 2014; Wheeler et al., 2017; Tabli et al., 2018). After the host is infected by *Pythium*, symptoms such as plant stunting, water-soak root rot, crown rot, and leaf blight could occur, resulting in host death (Kageyama et al., 2002; Higginbotham et al., 2004; Deng et al., 2005; Lin et al., 2018).

Pythium root rot can cause serious loss of agricultural production worldwide. In Kenya and Rwanda, *Pythium* root rot caused up to 70% yield losses in traditional local bean cultivars, while 35-54% and 12-17% loss at 18°C and 28°C in hydroponically grown lettuce Stanghellini and Kronland, 1986; Nzungize et al., 2012). Besides, *Pythium* root rot also reduced ginger (*Zingiber officinale* Rosc.) production by 50% to 90% in severely affected areas (Rai et al., 2018). Interestingly, Johnstone et al. (2005) reported that the net carbon exchange rate had no difference between inoculated and noninoculated bell pepper on leaf area basis, while inoculated treatment showed significantly lower on whole-plant basis. Moreover, the daily carbon gain showed a similar result with the net carbon exchange rate. These results indicated that *Pythium* species do not influence photosynthesis directly, but to reduce the biomass of the plant. This finding suggested that the symptoms caused by *Pythium* spp. at the very beginning will be difficult to notice.

Pythium spp. have a wide range of hosts. Crop rotation cannot control *Pythium*

efficiently due to its broad hosts. For example, four *Pythium* species isolated from symptomatic seedlings of corn and soybean showed equally pathogenic on both hosts (Rai et al., 2018). In another experiment, Feng et al. (2019) detected four plant-pathogenic *Pythium* from different tissue of lettuce by loop-mediated isothermal amplification (LAMP). From the Poaceae weeds grown next to the rice field in Akita Prefecture in Japan, *P. arrhenomanes* and *P. graminicola* had been isolated (Toda et al., 2015). Furthermore, 11 *Pythium* spp. isolated from bell pepper in Florida also caused root rot in tomato (Chellemi et al., 2000). In addition, a single host can be attacked by multiple species of *Pythium*. Chamswarng and Cook (1985) recovered ten species and varieties of *Pythium* from both wheat root and the soil in eastern Washington and northern Idaho. On the other hand, 11 different distinct of *Pythium* from both ginger and the soil caused ginger soft rot in Queensland, Australia (Le et al., 2016).

The longevity of *Pythium* also makes some traditional management methods ineffective. The *Pythium* can survive in the soil as oospores which has thick wall without host. Hoppe (1966) reported that *Pythium* species survived after 12 years in air-dried muck soil. The sporangia of *Pythium ultimum* geminated as usual after 11 months under air-dried conditions compared with the field soil maintain moistly, and the population afterward remained stable (Stanghellini and Hancock, 1971). Garren (1971) also found that *P. myriotylum* still aggressive after 10 months at room temperature in a sealed plastic bag with low inoculum. Moreover, Ichitani and Goto (1982) isolated *P. zingiberum* successfully from the field after crop rotation for more than 6 years in Japan. On the other hand, Samejima and Ichitani (1988) reported that oospores of *P. zingiberum* showed no gemination after 70 days buried in autoclaved soil with autoclaved cucumber stem segments, but the gemination ability of *P. butleri* had no change. Even in polar regions, mycelia of all tested *Pythium* in host plants survived after 3 freeze-thaw cycles (Murakami et al., 2015).

Given those above, *Pythium* can cause serious loss to many kinds of crops. Therefore, effective disease management is critically required. Using commercial fungicide could be a choice, however, people are anxious about the potential human

health risk and the fungicide resistant ability of *Pythium* enhanced by chemical compound (Margni et al., 2002; Lookabaugh et al., 2015). Considering that *Pythium* root rot is more common during summer and high moisture conditions in some area, some researchers are focusing on controlling *Pythium* spp. by temperature and moisture (Matthiesen et al., 2015; Wheeler et al., 2017; Huzar-Novakowiski and Dorrance, 2018). Besides, biocontrol is also a hot topic because of less human health and environmental risk (Van Os and Van Ginkel, 2001; Mavrodi et al., 2012). Furthermore, with the development of molecular biology, it had been proved that many signal pathways are involved in plant defense against *Pythium*, but the current knowledge is still not enough to help us to breed disease resistant plants (Adie et al., 2007; De Vleesschauwer et al., 2012; Sánchez-Vallet et al., 2012). The management of *Pythium* is complicated, since the aggressiveness of *Pythium*, the effectiveness of fungicide, the applicability of biocontrol agents and the application of disease resistant plants can be influenced by many factors such as air temperature, water pH, even soil type. Hereby, we had focused on common management strategies that can be used to minimize the economic loss caused by *Pythium*.

1. Physical and chemical control

Pythium species are widespread in soil and water (Senda et al., 2009; Toda et al., 2015; Uzuhashi et al., 2015). They can grow in a wide range of temperature, and the optimum temperature range from 13°C to 35°C depend on the species (Kageyama et al., 2002; Senda et al., 2009; Matthiesen et al., 2015; Huzar-Novakowiski and Dorrance, 2018). Plant root necrosis caused by *P. myriotylum* was highly correlated with nutrient solution temperature in hydroponic culture, while the lowest necrosis level at 15°C (Fortnum et al., 2000). Furthermore, other researchers also showed that low temperature could decrease the colonized and browned proportion in the root (Sutton et al., 2006). It is noteworthy that temperature not only influences the aggressiveness of *Pythium* spp., but also change the fungicide sensitivity of *Pythium*. Matthiesen et al. (2015) reported *P. oopapillum* and *P. torulosum* showed more aggressive under 18°C and 23°C, while

P. sylvaticum showed more aggressive under 13°C. Interestingly, *P. oopapillum* and *P. torulosum* were more sensitive to fungicide at the same temperature range which they were more aggressive.

Apart from temperature, water content is also a critical factor that affects symptom severity. Gent and McAvoy (2011) showed that partial saturation reduced biomass but did not affect the rate of flower development or plant nutrient composition. For instance, in three of five experiments, plants showed less root rot symptoms, and even the recovery rate of *Pythium* on agar was lower than standard saturation in some individual experimental groups (Elmer et al., 2012). Furthermore, by irrigating and draining rapidly to simulated partial saturation, the biomass and stem height reduced 10-20%, while no symptoms occurred under partial saturation (Gent and McAvoy, 2011). On the other hand, by controlling the water content, relatively low precise substrate volumetric water content (0.2 m^3/m^3) showed lower root infection by *P. aphanidermatum* in *Petunia ×hybrida* 'Dreams Red' compared with 0.4 m^3/m^3 and cyclic (0.18 to 0.43 m^3/m^3) treatment. However, the mortality proportion was lowest in cyclic treatment (Wheeler et al., 2017). Not only controlling water content, but also changing water property such as oxidation-reduction potential (ORP) inhibit *Pythium* root rot. For example, using chlorinated water eliminated *Pythium* zoospore in a few minutes (Lang et al., 2008). On the other hand, inoculated tomato grown under hydroponic conditions remained healthy with high oxygen concentration in solution (Chérif et al., 1997). Moreover, using filter and UV light are also considered as feasible way to control *Pythium*. However, it had been showed that filter only delayed root rot transmission in one or two week depending on the pore diameter, while UV light failed to relieve symptoms and reduced yield even if it could control zoospore population (Goldberg et al., 1992; Zhang and Tu, 2000).

There are many commercial chemical fungicides that are effective against *Pythium* spp. such as metalaxyl, azoxystrobin, fosetyl-Al, pyraclostrobin, and trifloxystrobin (Taylor et al., 2002; Stiles et al., 2005; Lookabaugh et al., 2015; Matthiesen et al., 2015). However, fungicides showed different abilities among different plants, even among

different *Pythium* spp. (Múnera and Hausbeck, 2015). Based upon environmental temperature, the EC_{50} value could increase more than 100 times in some fungicides (Matthiesen et al., 2015). Instead of chemical fungicides, some people set their sight on chemical agents which can enhance plant defense. For example, pre-treatment by Acibenzolar-S-methyl, a functional analogue of salicylic acid to turmeric (*Curcuma longa* L.) induce the activities of peroxidases and protease inhibitors, resulting in decreasing cell death after inoculation with *P. aphanidermatum* (Radhakrishnan et al., 2011). Apart from fungicide, Zhao et al. (2000) reported that the silver ion dissolved from silver-coated cloth reduced the root rot symptoms significantly. Surprisingly, nonionic surfactants completely controlled *Pythium* zoospores spread under hydroponic conditions (Stanghellini et al., 1996).

In addition to chemical products, some natural products such as *Brassica juncea* seed meal suppressed *P. abappressorium* constantly at least 12 weeks on apple seedlings (Weerakoon et al., 2012). Moreover, Gent and Elmer (2017) reported that combining with silicon, the disease symptoms of poinsettias (*Euphorbia pulcherrima*) significantly reduced under partial saturation ebb and flow system after inoculation with *P. aphanidermatum*. Meanwhile, polymer sodium silicate aqueous solution suppressed *Pythium in planta*, but not *in vitro* (Mohsen et al., 2015). Natural extract such as *Vitex agnus-castus* methanolic extract not only showed total antifungal ability against *P. ultimum in vitro*, but also induced certain pathogenesis-related proteins once inoculated with *Pythium* to enhance the plant defense ability, which showed unharmful to tomato seedlings (Švecová et al., 2013).

2. Biocontrol

In addition to chemical fungicides, biocontrol is also considered as a very promising way to control disease. By using the antagonism between different microorganisms, many biocontrol agents had been reported. For instance, *Enterobacter cloacae* and *Erwinia herbicola* were known to control preemergence damping-off effectively, and significantly suppressed *Pythium* colonization on cotton seed at 15°C

as effective as fungicide (Nelson, 1988). Misk and Franco (2011) reported that all 11 isolated endophytic actinobacteria from different plants showed antimicrobial ability against *P. irregulare*. Furthermore, strains isolated from irrigation well also showed *Pythium* damping-off controlling ability in pea (Tabli et al., 2018).

In addition, some biocontrol agents can even promote plant growth while relieving symptoms (Suwannarach et al., 2015). For example, six strains of *Pseudomonas* spp. were ideal biocontrol bacteria that reduced disease symptoms of both *Rhizoctonia solani* AG-8 and *P. ultimum* (Mavrodi et al., 2012). Besides, two strains of them even increased wheat seedling shoot length and root weight while suppressing pathogens (Mavrodi et al., 2012). Kipngeno et al. (2015) also reported that the dry mass of tomato seedling was significantly increased by coating *Bacillus subtilis* and *Trichoderma asperellum* to the seeds in the presence of fertilizer. Meanwhile, the post-emergence damping-off proportions were 10.87% and 15.3%, respectively, when compared that to the control (63.9%). On the other hand, some biocontrol agents can reduce symptoms through inducing plant defense systems. For example, *Pseudomonas corrugata* strain 13 and *Pseudomonas aureofaciens* strain 63-28 produced salicylic acid, and induced endogenous salicylic acid accumulation after inoculation with biocontrol agent for 24 hours in cucumber root (Chen et al., 1999). Furthermore, applying these two plant growth-promoting rhizobacteria suppressed cucumber root rot caused by *P. aphanidermatum* through stimulating the phenylalanine ammonia-lyase (PAL) activity, while *Pseudomonas corrugata* strain 13 also stimulated peroxidase (PO) and polyphenol oxidase (PPO) activities (Chen et al., 2000). In addition, by increasing benzyl isothiocyanate and its precursor glucotropaeolin in the root of Brassicaceae plant *Lepidium sativum* after inoculation with two non-pathogenic *Fusarium* strains, the resistance ability of host plant was enhanced against *P. ultimum* (Ishimoto et al., 2004). By microscopy, Benhamou and Brodeur (2001) found that a mycoparasite, *Verticillium lecanii* (Zimm.) not only inhibited the colonization of *P. ultimum*, but also induced host plant defense, to restrict the pathogen penetration in the epidermis and the outer cortex.

Keeping the population of the biocontrol agent is the key to ensure the

effectiveness of biocontrol. It was found that the biocontrol agent *Pseudomonas chlororaphis* Tx-1 will keep relative stable density after the sweet pepper was inoculated with *P. aphanidermatum* and *P. dissotocum*, but rapidly declined in the non-inoculated root (Chatterton et al., 2004). In another study, Postma et al. (2009) reported that combining with chitosan, the suppress ability of *Lysobacter enzymogenes* 3.1T8 against *P. aphanidermatum* in cucumber was enhanced and the bacterial population increased.

Except by using microorganisms directly, using compost is also an effective way to control *Pythium*. It was found that commercial compost strongly suppressed *Pythium* wilt disease on cucumber plants by the presence (0.056-0.36%) of fungal *Cystobasidiomycetes* and the presence (0.011-0.018%) of *Acidobacteria* Gp14 (Yu et al., 2015). On the other hand, the soil with higher organic matter content and lower sand and clay showed less disease index by comparing andosols and ferralsols in Cameroon (Adiobo et al., 2007). Moreover, the suppressiveness of andosols was significantly reduced by pasteurization, applying fungicide and bactericide. Similarly, using autoclaved rockwool increased disease incidence significantly compared with used or new rockwool due to more fungal population was present in used and recolonized rockwool (Postma et al., 2000). In addition, Van Os and Van Ginkel (2001) reported that *Pythium* growth rate was highest in sterilized soil, and lowest in sterilized soil with compost, suggested soil microflora can suppress *Pythium*.

3. Host plant defense

There is evidence that by expressing defense signaling pathways such as jasmonic acid (JA) signaling, mitogen-activated protein kinase (MAPK) signaling and wall-associated kinases (WAKs) inhibit root necrosis effectively after inoculation (Zhu et al., 2019). Furthermore, it had been reported that salicylic acid and gibberellic acid signal pathways are critical for rice (*Oryza sativa*) to defense against *P. graminicola*, while JA, abscisic acid, and ethylene signal pathway are involved in *Arabidopsis* defense against *P. irregulare* (Adie et al., 2007; De Vleesschauwer et al., 2012; Sánchez-Vallet

et al., 2012). Except for the critical signal pathway, Castro et al. (2016) found that constitutive expression of pathogenesis-related-10 gene in moss tissue increased resistance against *P. irregulare*. In another study, reducing availability of sugar in the rhizosphere due to a putative sugar transporter SWEET2 activity contributed to resistance to *P. irregulare* (Chen et al., 2015). Recently, Nair and Thomas (2013) found one potential resistant gene ZzR1 against *Pythium* in wild ginger relative *viz., Zingiber zerumbet* L. Smith.

Besides investigating the molecular aspect of plants, some researchers focus on breeding and screening. By examining the level of tolerance to *Pythium* root rot in main wheat product varieties, 'KS93U161', 'OH708', and 'Sunco' genotypes showed most tolerant to the disease determined by the number of root tips and total root length (Higginbotham et al., 2004). Besides, ornamental plants such as Caladium cultivars had also been screened and 4 of 19 cultivars showed partial resistance to *Pythium* root rot (Deng et al., 2005). Furthermore, by conducting somatic hybridization with potato 'Aminca-Cardinal' and 'Cardinal-Nicola', Nouri-Ellouz et al. (2006) obtained some hybrid lines which showed improved resistance ability against *P. aphanidermatum*, one line even noted as complete resistance. Among all the plants which showed tolerance against *Pythium*, phenolic compounds were identified as active ingredients. Temgo and Boyomo (2002) observed that the content of antifungal phenolic compounds determined the resistance ability of cocoyam clone.

Inducing the plant defense with exogenous stimulation is a hot topic during these decades. Many materials can be used to induce plant defense response. For instance, silicon can induce resistance in cucumber against *P. ultimum* by forming electron-dense layers along primary and secondary cell walls, and pit membranes of xylem vessels, as well as significantly increased the percentage of cells filled with phenolic-like material (Chérif et al., 1992). On the other hand, pre-treated with salicylic acid increased the activity of peroxidases and protease inhibitors, resulting in cell death reduction after inoculation with *P. aphanidermatum* (Radhakrishnan and Balasubramanian, 2009).

A classic example of using host plant defense is grafting, which is widely used in

the world to manage soil-borne disease. By grafting onto cucurbit hybrid rootstock 'Titan' and 'Hercules', no symptoms occurred on cucumber, and they also increased the vegetative growth and fruit product and quality comparing with self-grafted cucumber (Al-Mawaali et al., 2012).

Purpose

Li et al. (2007b) reported that *R. multiflora* 'Matsushima No. 3 showed great resistance ability against *P. helicoides*. Zhuang et al. (2012) further observed that the phenolic substances extracted from *R.* 'Matsushima No. 3' inhibited the germ tube elongation of *P. helicoides in vitro*. Preparing rose plant cutting and further domestication will at least take three months or more. Base on previous results, we try to investigate the total phenolic content in the plant root, to conduct a rapid preliminary screening.

MATERIALS AND METHODS

Plant materials and growth conditions

Plant cuttings for experiments are the same in Chapter I.

Standard sample preparation

gallic acid was used as standard. 0, 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, and 0.5 ml of Gallic acid solution (1.1 mg/ml) were prepared in flask. 1ml 0.5 N Folin-Ciocalteu (F-C) reagent (KANTO CHEMICAL CO., INC.) was added to each flask and shake for 30 s, then add 2 ml 700 mM Na_2CO_3 and volume up to 50 ml. Incubate the flask at room temperature under dark for 2 h after shaking gently.

Estimation of total phenolic content

Plant roots were harvested and freeze immediately in liquid nitrogen to homogenize the root tissue. Afterward, weigh the homogenized tissue (around 1 g, accurate to 0.01g) in 50 ml centrifuge tube, and add 45 ml of 70% ethanol in each tube. Place the tube under 40 Hz ultrasonic treatment (AS ONE, MCS-3) for 30 min, then water bath for 1 h at 40 °C. After water bath, centrifuge the samples (13,000×*g* for 5 min at room temperature) and collect 1 ml supernatant to 100 ml measuring flask, add 1 ml 0.5 N Folin-Ciocalteu (F-C) reagent shake for 30 s, then add 2 ml 700 mM Na_2CO_3 into each tube and volume up to 100 ml, incubate the flask at room temperature under dark for 2 h after shaking gently. Finally, use read the absorbance of each sample at 760 nm (SHIMADZU, UVmini 1240), then calculate the phenolic content by gallic acid standards. Each sample used at least two plants, and the absorbance was tested three times for each sample.

RESULTS

Figure 12 showed that the standard curve showed a linear relationship between absorbance and gallic acid concentration. Base on the standard curve, we obtained the total phenolic content of each sample. Figure 13 showed the phenolic content of each

plant. As we can see, M1-12 showed the highest total phenolic content, almost 15 mg/g FW, significantly higher than all other individuals. M1-15 and M-9 showed 13.2 and 12.9 mg/g FW, respectively, ranked in the 2nd echelon. Tetraploid of *R. multiflora* 'Matsushima No. 3' only showed 8.8 mg/g FW phenolic content in this experiment, which is the second lowest among all individuals.

DISCUSSION

Root rot caused by *Pythium* brought great economic loss in rose production in Japan. It had been reported that the phenolic content extracted from tetraploid of *R. multiflora* 'Matsushima No. 3' inhibited the germ tube elongation of *P. helicoides in vitro*, and HPLC also confirmed that the bound phenolic content in tetraploid of *R. multiflora* 'Matsushima No. 3' is higher than the susceptible variety (Zhuang et al., 2012). In this experiment, we used F-C reagent to estimate the total phenolic content in the root. Figure 14 showed that most of the tested M1 backcross line had a higher phenolic content than tetraploid of *R. multiflora* 'Matsushima No. 3', suggested the root rot resistance could be better. Tetraploid of *R. multiflora* 'Matsushima No. 3' only had 8.8 mg/g FW phenolic concentration in the root, which was the 2nd lowest among all individuals. However, this method cannot distinguish free and bound phenolic compounds. It had been reported that the accumulation of cell-wall bound phenolic compounds can significantly reduce the root rot symptoms (Chérif et al., 1994; Takenaka et al., 2003). Thus, it is possible that the bound phenolic content in the root of tetraploid of *R. multiflora* 'Matsushima No. 3' is higher. The previous report also indicated that the tetraploid of *R. multiflora* 'Matsushima No. 3' had no resistance mechanisms at the early process of infection, suggested the tolerance mechanisms could be induced by *Pythium*. However, in this experiment, limited by the low rooting rate of plant cutting and the low zoospore yield when prepared inoculum, we did not conduct the infection experiment. For further research, the problem that needs to be solved urgently is to find a method that can ensure the rooting rate, and the zoospore production.

Besides, it should be better to investigate if the phenolic content can be induced or not after inoculation.

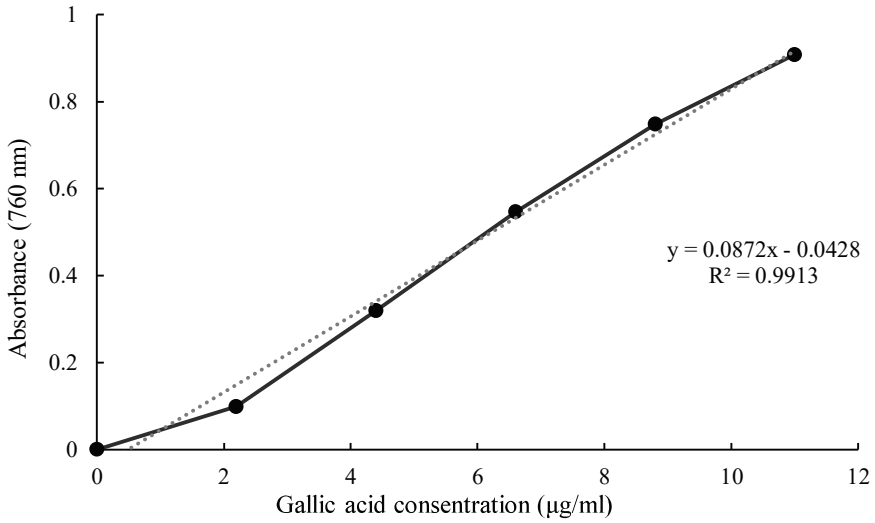

Figure 12. Gallic acid standard curve use to calculate the phenolic content.

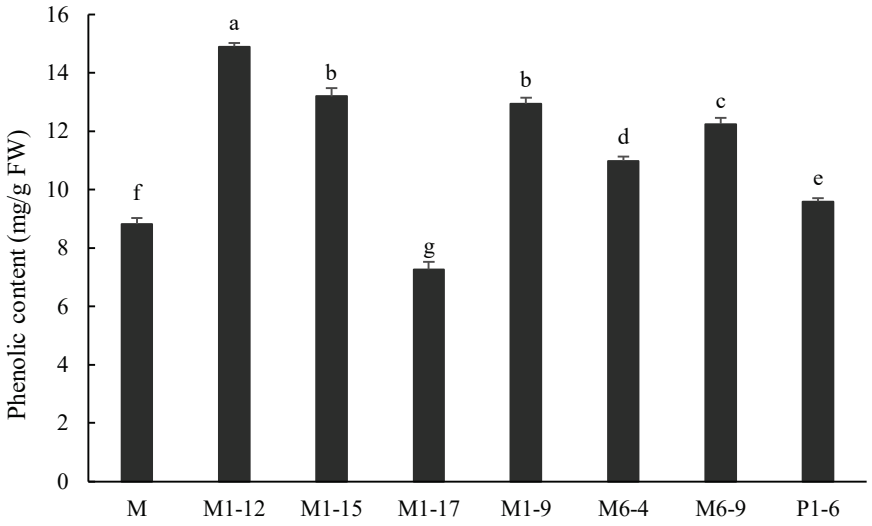

Figure 13. Total phenolic content of each plant root. Different letters indicate significant differences at $P < 0.05$ level by using Tukey's HSD. Bars show mean values (±SD) of three independent experiments. “M” indicates tetraploid of *R. multiflora* ‘Matsushima No. 3’.

CONCLUSION

As a standard of evaluating crown gall disease symptoms on the plant, needle prick had been widely used in crown gall disease research on rose and progeny selection. Meanwhile, the evaluating method for root rot disease is to check the browning rate of the root after infection. However, these methods require a large number of samples, and a lot of time to prepare the plant depending on the plant characters. This experiment aimed to find a rapid method, to accelerate the breeding progress. For crown gall disease, current findings suggest that the opine assay was limited by the callus formation rate of the individuals. To get rid of this limitation, we observed the oncogene expression level after infection. The results suggested that the oncogene expression showed a relationship with the results of needle prick. Among the backcross line, the individuals which showed lower oncogene expression level showed smaller wounds than the higher individuals in the needle prick experiment. Thus, screening the crown gall disease resistance among rose progenies by using oncogene expression analysis is feasible. For the root rot disease, we evaluated the total phenolic content in the plant root without inoculation. The results indicated that most M1-x backcross lines showed higher phenolic content, suggested the potential resistance ability against root rot disease. For further research, we can use these methods for a rapid screening at the very beginning, then prepare the cutting of putative resistance varieties to conduct field test.

Questions need to be answered

So far, the resistant mechanisms of *R.* 'PEKcougel' were considered as the exudate from the wound that blocked the attachment of bacteria (Tan et al., 2004). To further prove the success of T-DNA transfer, using genetic modification T-DNA that contains β-glucuronidase (GUS) or green fluorescent protein (GFP) as a reporter can clear this. On the other hand, the mechanism of 'Matsushima No. 3' could relate to resistance to tumor development or formation. As for phenolic content, bound phenolic induced by *Pythium* may be related to root rot disease resistance of 'Matsushima No. 3'.

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