Evaluation of Crown Gall Disease Resistance in Hybrids of *Rosa* ‘PEKcougel’ and Tetraploid of *R. multiflora* ‘Matsushima No. 3’

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Crown gall disease caused by *Agrobacterium tumefaciens* causes economic loss in ornamental production in Japan. To breed a resistant rootstock, we used crown gall resistant *Rosa* ‘PEKcougel’ to hybridized with tetraploid of *R. multiflora* ‘Matsushima No. 3’. In this study, we investigated the crown gall resistance characteristics in their progenies. In the needle prick test, eight tested progenies showed significantly smaller wounds than *R*. ‘PEKcougel’. In the stem segment culture and opine assay, six progenies formed no callus tissue while three progenies formed calluses and had no opine detected. Further oncogene expression analysis was performed to evaluate crown gall disease resistance, since the low callus formation rate limited the application of opine assay. In the oncogene expression analysis, some progenies of tetraploid of *R. multiflora* ‘Matsushima No. 3’ showed a lower expression level of both *ipt* and *iaaM* than *R*. ‘PEKcougel’. Furthermore, *ipt* expression had a significant positive relationship with tumor size. Taken together, these results suggest that *ipt* expression analysis can be used for evaluating crown gall disease resistance in rose progenies. Furthermore, eight progenies with strong crown gall disease resistance were confirmed.

Key Words: *Agrobacterium tumefaciens*, *iaaM*, *ipt*, needle prick, oncogene expression.

Introduction

Crown gall disease caused by *Agrobacterium tumefaciens* is a common disease which occurs in many types of fruit trees, grapevines, and some ornamental crops (Bliss et al., 1999). *A. tumefaciens* is a soil-borne pathogen that has a wide host range and can mediate gene transfer during infection (De Cleene and De Ley, 1976). Generally, crown gall disease is not lethal for plants; however, it can cause great economic losses due to quality degradation and yield reduction (Poncet et al., 1996). Roses are one of the most important ornamental crops around the world affected by crown gall disease (Zhou et al., 2000).

Since the pathogen of crown gall disease was discovered, its management generally focuses on phytosanitation (Escobar and Dandekar, 2003). Recently, biocontrol has been shown to be an effective way to control crown gall disease. It has been reported that many nonpathogenic *Agrobacterium* can be used as biocontrol agents such as *A. radiobacter* K84, *A. vitis* VAR03-1, *A. vitis* ARK-1, and even the genetically engineered *Agrobacterium* strain, K1026 (Kawaguchi et al., 2008, 2015; McClure et al., 1998; Penyalver et al., 2000). Although these biocontrol agents have been shown to be effective against pathogens, the strain of pathogen and the plant species significantly affect the effectiveness of the biocontrol agents (Kawaguchi et al., 2015). Thus, it is still necessary to breed disease-resistant crops.

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 the T-DNA fragment, the genes which are critical for crown gall disease can be divided into two groups, oncogenes and opine genes (Bourras et al., 2015). Among the oncogene fragments, three genes are critical for cell proliferation and crown gall development: tryptophan-2-monoxygenase (*iaaM*), indole-3-acetamide hydrolase (*iaaH*), and isopentenyl transferase (*ipt*) genes. The *iaaM* and *iaaH* genes encode enzymes for the biosynthesis of auxin and *ipt* encodes an enzyme for cytokinin.
(Klee et al., 1984; Lichtenstein et al., 1984). The expression of oncogenes can lead to plant hormone overproduction, shown as uncontrolled callus growth, and *iaam* and *ipt* are critical for this process (Escobar et al., 2001). The expression of gene pairs after T-DNA transfer can provide a carbon/nitrogen source for *A. tumefaciens* (Coenen and Lomax, 1997). The resistance mechanism of plants against *A. tumefaciens* can be divided into two parts. The first is to prevent T-DNA transfer and the second is to regulate tumor formation and development after infection (Anand et al., 2007; Hansen, 2000; Narasimhulu et al., 1996; Tan et al., 2004).

Crown gall disease caused by *A. tumefaciens* impedes rose production in Japan (Zhou et al., 2000). It has been reported previously that *Rosa* ‘PEKcougel’ showed resistance to crown gall disease by blocking the attachment of bacteria to the wound surface with exudates, while the highly susceptible *R.* ‘Dukat’ and *R. multiflora* ‘Matsushima No. 3’ showed no such resistant mechanism (Li et al., 2008; Tan et al., 2004; Zhou et al., 2000). *R. multiflora* ‘Matsushima No. 3’ showed root rot disease resistance caused by *Pythium helicoides* (Li et al., 2007). Moreover, in a preliminary experiment, we found that tetraploid of *R. multiflora* ‘Matsushima No. 3’ did not form typical tumor tissue. Therefore, we used *R.* ‘PEKcougel’ hybridized with tetraploid of *R. multiflora* ‘Matsushima No. 3’ to breed a rootstock with crown gall and root rot resistance that can help to manage these diseases. Crown gall resistance is commonly evaluated by the needle prick test; however, traditional screening requires a large amount of plant material and takes a lot of time (Boelema, 2004). The expression of opine genes after T-DNA transfer can provide a carbon/nitrogen source for *A. tumefaciens* (Klee et al., 1984; Lichtenstein et al., 1984). The expression of opine assay, and measured the expression of oncogenes, then assessed their usefulness for evaluating crown gall resistance in rose progenies.

### Materials and Methods

**Plant materials and growth conditions**

The resistant variety *Rosa* ‘PEKcougel’, tetraploid of *R. multiflora* ‘Matsushima No. 3’, their F₁ and BC₁ generations, and the susceptible variety *R.* ‘Dukat’ were used for crown gall disease screening. Tetraploid of *R. multiflora* ‘Matsushima No. 3’ was obtained by colchicine treatment in 2009. The ploidy level was checked by flow cytometry. Information on the F₁ and BC₁ generations are shown in Table 1. The F₁ generation was obtained by crossing with *R.* ‘PEKcougel’ as a seed donor and tetraploid of *R. multiflora* ‘Matsushima No. 3’ as a pollen donor. Among the BC₁ generation, only the plants which were in good condition and had young shoots were used. Up to 30 of 73 individuals obtained by hybridization were tested. Needle prick inoculation was conducted using rooted cuttings. The stems were cut into small segments that contained one lateral bud. The segments were placed in water-saturated mineral wool and sealed in a plastic bag. The bag was placed under 25°C with 24 h light (160 μmol·m⁻²·s⁻¹) for 4 weeks for rooting. Afterward, the rooted individuals were transferred to a germination tray full of germination soil BM2 (Berger, Canada), and placed under 25°C, 45–60% humidity, and 24 h light (160 μmol·m⁻²·s⁻¹) for acclimatization for 1–2 months before inoculation. The growth conditions remained the same after inoculation. The symptoms were observed 5 weeks after inoculation. The horizontal diameter of each inoculated site was measured by vernier caliper. Fold changes of the inoculated sites were calculated by the average horizontal diameter of the inoculated site divided by the average horizontal diameter of the mock. To conduct stem segment culture for opine assay and qRT-PCR, shoots between leaves expanding and expanded were harvested and washed with neutral detergent, then rinsed with tap water for 20 min. Afterward, the shoot was rinsed with 70% ethanol for 30 s, then surface sterilized for 20 min with 1% (active chlorine v/v) sodium hypochlorite containing 0.01% (v/v) Tween-20. The shoots were cut into 5 mm lengths and 2 mm lengths for opine assay and qRT-PCR, respectively. After cutting the shoots, the stem segments were soaking in sterilized distilled water (dH₂O) for 5 min followed by inoculation. After inoculation, the stem segments were grown on hormone-free Murashige and Skoog (MS) medium (30 g·L⁻¹ sucrose, 2 g·L⁻¹ gellan gum, pH 5.7) for 72 h in a growth chamber with 16 h light (120 μmol·m⁻²·s⁻¹) at 25°C. Subsequently, the stem segments were shaken and washed with cefoo-

| Name     | Generation | Seed donor       | Pollen donor       | Obtained number |
|----------|------------|------------------|--------------------|-----------------|
| F₁ No. x | F₁         | *R.* ‘PEKcougel’ | Tetraploid of *R. multiflora* ‘Matsushima No. 3’ | 3               |
| P1-x     | BC₁        | *R.* ‘PEKcougel’ | F₁, No. 1          | 11              |
| P6-x     | BC₁        | *R.* ‘PEKcougel’ | F₁, No. 6          | 4               |
| M1-x     | BC₁        | Tetraploid of *R. multiflora* ‘Matsushima No. 3’ | F₁, No. 1        | 36              |
| M6-x     | BC₁        | Tetraploid of *R. multiflora* ‘Matsushima No. 3’ | F₁, No. 6        | 19              |

* Current existing individuals obtained through hybridization.
formic acid, acetic acid, and dH$_2$O moved by the phenol/chloroform extraction method. The paper was observed with ultraviolet light (312–360 nm) after it was dried. RNA extraction and gene expression analysis was conducted following the manufacturer’s protocol. The extracted total RNA was used for Opine assay and qRT-PCR, the segments were soaked in bacterial solution for 5 min. Replicates were performed three times for qRT-PCR, while the opine assay was calculated with the total tested number considering the significant difference in fold change of the inoculated site and oncogene expression.

Pathogen preparation and inoculation

*A. tumefaciens* ‘GOU1’ was as the pathogen (Zhou et al., 2000). The pathogen was first cultured on solid yeast extract beef (YEB) medium (5 g·L$^{-1}$ beef extract, 1 g·L$^{-1}$ yeast extract, 5 g·L$^{-1}$ peptone, 5 g·L$^{-1}$ sucrose, 493 mg·L$^{-1}$ MgSO$_4$·7H$_2$O, and 15 g·L$^{-1}$ agar, pH 7.2) under darkness at 25°C for 48 h. Before inoculation, the pathogen was transferred to YEB liquid medium and cultured under darkness at 25°C and 120 rpm for 24 h. Finally, the inoculum was adjusted to approx. 2 × 10$^7$ CFU·mL$^{-1}$ by optical density (OD$_{600}$). As for needle prick inoculation to the rooted cuttings, the new shoot that grew from the lateral bud was used to conduct inoculation using the needle prick inoculation method using a needle with a diameter of 0.6 mm (Tolba and Zaki, 2011). Depending on the shoot length, at least five places were pricked with an inoculation needle to place a drop of inoculum at the cambium. At least 3 cuttings were used. Sterilized dH$_2$O was used for mock inoculation. As for stem segment inoculation for opine assay and qRT-PCR, the segments were soaked in bacterial solution for 5 min. Replicates were performed three times for qRT-PCR, while the opine assay was calculated with the total tested number considering some individuals were unable to provide enough shoots.

Opine assay

The callus tissue from the 5 mm length stem segments was carefully isolated and squared in a 1.5 mL tube 3 weeks after inoculation. Then, 3 μL supernatant was spotted on chromatography paper (GE Healthcare Life Science, USA) for electrophoresis (myPower II 500 AE-8155; ATTO, Japan) with a buffer made from formic acid, acetic acid, and dH$_2$O (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$^{-1}$ in 99.5% ethanol) mixed with sodium hydroxide solution (0.1 g·mL$^{-1}$ in 60% ethanol) as 1:1 (v/v)). Finally, the paper was observed with ultraviolet light (312–360 nm) after it was dried.

RNA extraction and gene expression analysis

The total RNA from the 2 mm stem segments was extracted 1 week after inoculation using an RNA extraction kit (RNA suisui-R; Rizo, Japan) following the manufacturer’s protocol. The extracted total RNA was digested with DNase I (Nippon gene, Japan) for 25 min at 37°C to remove genomic DNA. DNase I was removed by the phenol/chloroform extraction method. cDNA synthesis was performed using a PrimeScript™ RT reagent Kit (Perfect Real Time) (TakaraBio, Japan). Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TakaraBio). PCR was performed as follows: an initial denaturation of 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 60.5°C for 45 s. A dissociation analysis step (95°C for 15 s, 60.5°C for 30 s, and 15 s at 95°C) was added after the cycle reaction. The forward primer 5′-AAATTACTGGC TTGGCCCCT-3′ and reverse primer 5′-TTTGGCGATC CACATCTGCT-3′ were used for *ACT4* (housekeeping gene) amplification. The forward primer 5′-AGCCGTC TATACCTTGATGATCG-3′ and reverse primer 5′-TCTATCGC TGAAGAGAGGACTC-3′ were used for *ipt* amplification. The forward primer 5′-CGCCTTGTGCT ATTACTCAAGC-3′ and reverse primer 5′-TCCTACGC TGAAGAGAGGACTC-3′ were used for *iaaM* amplification. The relative expression level of *ipt* and *iaaM* were normalized against *ACT4*. *R. ‘PEKcougel’* was set as calibrator.

Data analysis

Tukey’s HSD test ($P<0.05$) was used to determine the significant difference in fold change of the inoculated site and oncogene expression. Pearson’s correlation coefficient was used to evaluate the linear relationship between oncogene expression and fold change of the inoculated site, and oncogene expression and the opine detection rate. *R. ‘Dukat’* was not used in the statistical analysis because it had no relationship with the hybrid progenies.

Results

Crown gall resistance evaluation by needle prick

The appearance and the fold change of the inoculated sites are shown in Figures 1 and 2. The susceptible variety *R. ‘Dukat’* showed a bigger fold change in the inoculated site than the other tested samples (Fig. 2). Among the tested samples, *R. ‘PEKcougel’* showed the biggest fold change in the inoculated site, significantly greater than the other tested individuals. The fold changes 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 changes 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 was difficult to conduct the needle prick test on the other progenies. Thus, we tried inoculation of *A. tumefaciens* using stem segment culture, opine assay, and oncogene expression analysis to evaluate the resistance against crown gall disease.

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 2). The susceptible variety *R.* ‘Dukat’ showed a higher opine detection rate than *R.* ‘PEKcougel’. The opine detection rate of F₁ No. 1 was lower than *R.* ‘PEKcougel’. F₁ No. 6 had a 66.7% opine detection rate. However, this could be inaccurate due to the low callus number. Among all the tested progenies, the M1 line and M6 line showed a lower callus formation rate than the P1 line and P6 line. Furthermore, 6 individuals from the BC₁ line (M1-5, M1-23, M1-25, M1-30, M1-33, and M6-2) formed no callus tissue, and all these individuals were from the backcross line of tetraploid of *R. multiflora* ‘Matsushima No. 3’ with F₁ generation. Among the tested individuals which formed a callus, 3 individuals (M1-12, M1-15, and M6-10) showed no opine detected. As for the tested individuals which formed no callus, further screening is necessary. Therefore, M1-5, M1-23, M1-25, M1-30, M1-33, and M6-2, which formed no callus, were used for the next oncogene expression analysis. Moreover, individuals with a callus formation rate lower than 20% or lower opine detection rate than *R.* ‘PEKcougel’ were used for gene expression analysis.

**Oncogene expression analysis**

Both the *ipt* and *iaaM* expression levels of *R.* ‘Dukat’ were higher than *R.* ‘PEKcougel’ (Fig. 3). Two BC₁ individuals (P1-4 and M6-7) and *R.* ‘PEKcougel’, which showed a greater fold change of the inoculated site in the needle prick test, also had a higher expression level of *ipt*. The remaining individuals had a significantly lower expression level in comparison. Furthermore, the relationship between oncogene expression and tumor size or the opine detection rate was investigated. The results showed that the *ipt* gene had a positive correlation (*r* = 0.58) with a significant relationship to the fold change of the inoculated site (Fig. S1). On the other hand, the correlation coefficient between the opine detection rate and the expression of both oncogenes were *r* = 0.19, and had no significant relationship.
between the opine detection rate and the expression of oncogenes (Fig. S2).

**Discussion**

At the first screening, the needle prick test was performed to analyze crown gall disease resistance in the rose progenies. We found that tetraploid of *R. multiflora* ‘Matsushima No. 3’ had a significantly lower fold change in the inoculated site than *R.* ‘PEKcougel’, while the fold change of the inoculated site in most of the backcross progenies was between tetraploid of *R. multiflora* ‘Matsushima No. 3’ and *R.* ‘PEKcougel’ (Fig. 2). Furthermore, the susceptible variety *R.* ‘Dukat’ had a bigger fold change in the inoculated site than *R.* ‘PEKcougel’ (Figs. 1 and 2), which is consistent with a previous report (Zhou et al., 2001). Interestingly, some backcross progenies of tetraploid of *R. multiflora* ‘Matsushima No. 3’ did not form tumor tissue, suggesting that the resistant mechanism of tetraploid of *R. multiflora* ‘Matsushima No. 3’ is different from *R.* ‘PEKcougel’. For instance, Nam et al. (1997) found that resistance to crown gall disease development was a result of random mutations inherent in the transformation system. Mahmoodzadeh et al. (2004) also found that a hybrid grapevine had a smaller gall size and lower percentage of galls representing strong resistance. These indicate the complexity of disease resistance mechanisms in progenies.

However, the needle prick test requires a large amount of plant material and is time-consuming, and is limited by the rooting percentage. Opine assay has been shown to be an effective method to distinguish tumor tissue from callus tissue (Escobar and Dandekar, 2003). Thus, we tried to apply opine assay to infected stem

| Variety or line | Total sample number | Callus number | Opine detected number | Callus formation rate (%) | Opine detection rate (%) |
|----------------|---------------------|---------------|-----------------------|---------------------------|--------------------------|
| P              | 39                  | 17            | 7                     | 43.6                      | 41.2                     |
| M              | 20                  | 0             | —                     | 0                         | —                        |
| F1, No. 1      | 79                  | 20            | 6                     | 25.3                      | 30.0                     |
| F1, 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          | 33                  | 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                     |

Callus formation rate (%) = Callus number/Total sample number × 100
Opine detection rate (%) = Opine detected number/Callus number × 100

“P” represents the resistant variety *R.* ‘PEKcougel’, “M” represents tetraploid of *R. multiflora* ‘Matsushima No. 3’, and “D” represents the susceptible variety *R.* ‘Dukat’.

Opine detection rate was not calculated when the callus number is less than three.
segment culture to evaluate disease resistance. After T-DNA was inserted into the plant genome through Agrobacterium infection, the transformed plant cell will produce opines due to the existence of opine-related genes as a substrate for Agrobacterium (Pedersen et al., 1983; Yang et al., 1987). However, in this study, we found that almost one third of tested individuals formed no callus tissue, and some individuals showed a quite low callus formation rate or formed small callus tissue, which limited the application of opine assay (Table 2).

Currently, several mechanisms of plant resistance against A. tumefaciens have been reported, including a lack of vir-inducing factors (e.g., acetosyringone derivatives) and T-DNA integration factors, plants containing antibiotic compounds, as well as suppressing oncogene expression in transformed cells (Licauzi et al., 2019; Narasimhulu et al., 1996; Sahi et al., 1990; Tan et al., 2004; Zhang et al., 2015). For instance, silencing ipt and iaaM in Arabidopsis and tomato can enhance 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 an important index to evaluate the resistance against crown gall disease in rose. In this study, we found that the individuals which showed both a higher expression of ipt and iaaM had a greater fold change in the inoculated site than the others (Figs. 2 and 3). These results indicate the complexity of crown gall disease resistance mechanisms (Bourras et al., 2015; Escobar and Dandekar, 2003; Kim and Park, 2019). Furthermore, within the BC1 backcross lines and their parents, a significant positive correlation was found between ipt expression and the fold change of the inoculated site, suggesting that the ipt expression analysis can be used for crown gall disease resistance evaluation by needle prick inoculation (Fig. S1A). However, since iaaM expression showed no significant relationship with the fold change of the inoculated site, the effectiveness of using iaaM expression to evaluate disease resistance needs further study (Fig. S1B). On the other hand, the opine detection rate and oncogene expression did not show any relationship. This could be explained by the reason that oncogenes are related to the tumor formation and development process, while opines produced by the plant only relate 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 had a high opine detection rate with low oncogene expression, while some individuals had high oncogene expression but with a low opine detection rate (Fig. S2). This result was consistent with previous findings (Escobar and Dandekar, 2003; Subramoni et al., 2014). M1-45 showed high iaaM expression while the needle prick result showed resistance, and M6-2 showed a relatively high fold change of inoculated sites with low oncogene expression. The reason for this result may due to the growth promoting effect of cytokinin and auxin after infection that varies dependent on the 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 among plant species (Escobar et al., 2001, 2002; Lee et al., 2003; Viss et al., 2003; Zhang et al., 2015). Taken together, these results suggest that ipt expression analysis can be used to evaluate the progression of tumor formation and development, while opine assay can only be used to evaluate whether the plant was infected.

Overall, within hybrid progenies, the crown gall resistance of P6-4, M1-9, M1-12, M1-15, M1-23, M1-30, M1-45, and M6-4 were greater than R 'PEKcougel. These individuals can be used for further study to clarify their resistance mechanisms and their ability to be used as rootstock.

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