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Note

Stability of pUC-Derived Plasmids with a Fluorescence Marker in *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *betavasculorum*

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The stability of three different kinds of pUC-derived plasmids, pDsRed, pZsYellow, and pGFPuv, was investigated in *Pectobacterium* strains to utilize those plasmids as tracers. All three plasmids pDsRed, pZsYellow and pGFPuv showed their specific colors in *Pectobacterium* strains. Especially, the plasmid pDsRed conferred bright pink colonies on the *Pectobacterium* strains. When the bacteria lost the plasmid pDsRed, the colonies turned white, suggesting that the plasmid could be a good marker system for *Pectobacterium* strains on different environmental conditions. The effect of the antibiotic pressure on the stability of the plasmid was different depending on the host bacteria. *P. carotovorum* subsp. *betavasculorum* was more sensitive to the antibiotic pressure than *P. carotovorum* subsp. *carotovorum* Pcc21. However, temperature change significantly affected plasmid stability on both *Pectobacterium* strains. Almost all strains lost the plasmids with the shift in temperature from 28°C to 37°C. Presence of the plasmids did not affect bacterial pathogenicity on their own host plants. Among three plasmids, pZsYellow was not useful as a marker because the yellow fluorescent proteins from pZsYellow were interfered with the yellow natural fluorescence of the plant tissues induced by the defense system. Since the red color of DsRed can be seen with naked eyes, plasmid pDsRed was applicable as a marker. However, the color change was slow so that additional manipulation to increase the expression speed was necessary. Plasmid pGFPuv could serve as a perfect marker without any problem, tracing the reproduction and spread of the plant pathogens perfectly.

Keywords : pDsRed, *Pectobacterim carotovorum*, pGFPuv, pUC-derived plasmids, pZsYellow

Enterobacteriaceae is a large family of bacteria and it includes many familiar genera such as *Escherichia*, *Salmonella* and *Pectobacterium*. *Escherichia* contains a well-known bacterium *Escherichia coli*. Previously known as *Erwinia*, *Pectobacterium* genus contains various plant pathogenic bacteria such as *Pectobacterium carotovorum* subspecies, *Pectobacterium chrysanthemi*, and *Pectobacterium betavasculorum*. Especially *P. carotovorum* subsp. *carotovorum* causes destructive soft rot disease on most vegetables, so it is one of the major research subjects of the latest studies. Recently, reorganization of *Pectobacterium* genus was suggested for the subspecies strains of *Pectobacterium carotovorum*, previously being *P. carotovorum* subsp. *carotovorum*, subsp. *atrocepticum*, subsp. *betavasculorum*, subsp. *odoriferum*, and subsp. *wasabiae* into *P. carotovorum*, *P. atrocepticum*, *P. betavasculorum*, *P. odoriferum*, and *P. wasabiae* (Gardan et al., 2003). *P. carotovorum* subsp. *carotovorum* and subsp. *chrysanthemi* are pectolytic bacteria that cause soft rot, which makes the plant tissues totally softened, on various kinds of plants. However, three other subspecies show little different phenotypes from the soft rot caused by subsp. *carotovorum* and *chrysanthemi*. *P. carotovorum* subsp. *betavasculorum* causes vascular necrosis exclusively on sugar beet and sunflower, subsp. *wasabiae* is responsible for internal discoloration of rhizomes of wasabi, and subsp. *odoriferum* is responsible for slimy rot of chicory. *P. carotovorum* subsp. *carotovorum* is a notorious pathogen on Chinese cabbage. Like many other enteric pathogens, such as *E. coli* and *Yersinia* sp., *P. carotovorum* subsp. *carotovorum* and *chrysanthemi* do not appear to invade host cells. They remain in the intercellular spaces of infected plant tissue and use several secretion systems to inject virulence factors into the host cells. In addition to causing local disease, the bacteria may enter vascular elements of infected plants, thereby moving rapidly through the host (Spinelli et al., 2005).

Fluorescent proteins have revolutionized many areas of cell biology and biotechnology because they provide strong visible fluorescence. Currently, there are many different kinds of fluorescent proteins available. The most frequently used one is the green fluorescent protein (GFP) originated from the jellyfish *Aequorea victoria*. It has been the most revolutionary reporter in biology since its application as a marker was published by Chalfie et al. (1994). The major advantage of using GFP as a trace marker is the noninva-

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sive analysis without exogenous application of substrates or energy. Therefore, GFP can be an effective marker in observing plant microbe interaction. Many kinds of GFP variants have been produced to provide stronger fluorescence and broaden the range of applications. Even GFP variants with blue, cyan, and yellowish green emissions are now available. Red fluorescent protein (DsRed), which was discovered later than the GFP, comes from the coral *Discosoma* genus (Baird et al., 2000). Protein DsRed is a 28 kDa fluorescent protein responsible for the red coloration around the oral disk of the coral of the *Discoma* genus. DsRed has attracted tremendous interest as a potential expression tracer because of its distinct color from GFP that can be seen in the naked eyes. The yellow fluorescent protein, which comes from another coral *Zoanthus* species, was engineered for a brighter fluorescence and started to be used in recent days (Matz et al., 1999).

Plasmid containing pUC origin is a commonly used type of cloning vector and expression vector (Yanisch-Perron et al., 1985). It is usually accompanied by lac promoter of *E. coli,* so that it has good capability of being replicated in *E. coli* strains. The presence of fluorescent plasmids usable for *Pectobacterium* species will definitely promote the researches on the *Pectobacterium* species. It is not well known whether pUC origin plasmids can serve as good reproductive markers in *Pectobacterium* strains. It is expected that those plasmids will function well because genera *Escherichia* and *Pectobacterium* are in the same family.

In this study, we monitored the stabilities of pUC-derived plasmids in *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *betavasculorum*, causing two distinct phenotypes, soft rot and necrosis. This study was conducted to find out whether pUC-derived plasmids carrying fluorescens marker can serve as good tracers of *Pectobacterium* strains.

Expression of fluorescens in *Pectobacterium carotovorum* subsp. *carotovorum* and subsp. *betavasculorum* using a pUC-derived plasmid

Since *Pectobacterium* spp. belong to *Enterobacteriacea* family together with *E. coli*, it is suggested that the plasmids stable in *E. coli* may replicate well in *Pectobacterium* spp., too. If the narrow host range plasmids such as pUC can replicate well in *Pectobacterium* spp., it can help the *Pectobacterium* research greatly. In order to investigate the stability of pUC plasmids in *Pectobacterium* spp., *P. carotovorum* subsp. *carotovorum* Pcc21 (Roh et al., 2008) and *P. carotovorum* subsp. *betavasculorum* (Gardan et al., 2003) were chosen. Since those two distinct strains have very different host ranges, they can be good candidates to conduct an ecological adaptation study. *P. carotovorum*

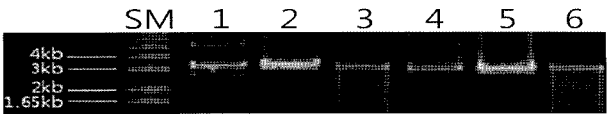

Fig. 1. Agarose gel electrophoresis of plasmids of *Pectobacterium*. Re-isolated plasmids from transformants were treated with *Eco*RI. Lane 1, 2 and 3 showed the plasmids isolated from the colonies of *P. carotovorum* subsp. *carotovorum* Pcc21 and 4, 5, and 6 showed the plasmids isolated from the colonies of *P. carotovorum* subsp. *betavasculorum*. Lane 1 and 4 are plasmid pDsRed, 2 and 5 are plasmid pZsYellow, and lane 3 and 6 are the plasmid pGFPuv.

subsp. *carotovorum* can cause soft rot disease on diverse vegetables including cabbage, carrot and radish. However, *P. carotovorum* subsp. *betavasculorum* can cause necrosis on mainly sugarbeet. Also, the mechanism of the disease occurrence is very different.

Three different fluorescent plasmids pDsRed, pZsYellow and pGFPuv (Clontech, Mountain View, USA) were selected to be transformed into *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum.* Those plasmids were easily transformed into the strains and the transformation ratio was very high (data not shown). After the confirmation of transformants with PCR (Fig. 1), the stability of three plasmids in *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum* was investigated with the fluorescens expression in each selected transformant. All three plasmids expressed their colors well in both *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum* (Fig. 2). Plasmids pGFPuv and pZsYellow exhibited their colors as fast as the colonies grow. However, it was found that the red fluorescent protein in pDsRed was not expressed enough until the pigments were accumulated for two days. It took three days for all colonies to turn red.

In addition to the delay of the color development in bacterial transformants carrying plasmids pDsRed, the color of several transformants was not changed into red (data not shown). To verify the presence of the plasmid pDsRed in the white transformants of *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum*, plasmids were isolated from 20 white or red transformants of *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed) and *P. carotovorum* subsp. *betavasculorum* (pDsRed); Also, PCR amplification of total DNA isolated from 20 white or red transformants of *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed) and *P. carotovorum* subsp. *betavasculorum* (pDsRed) was conducted. All white colonies did not carry plasmid pDsRed and also did not have any homologous DNA with pDsRed based on PCR (Fig. 3). However, this phenomenon was not observed in the transformants of pGFPuv or pZsYellow at all, suggest-

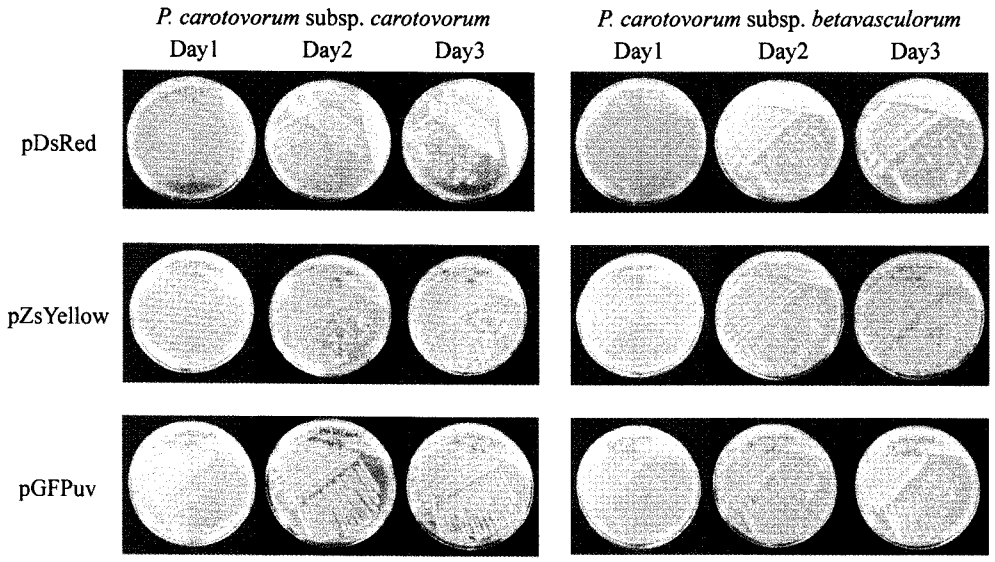

Fig. 2. The fluorescent light emitted from the three different markers in *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum.*

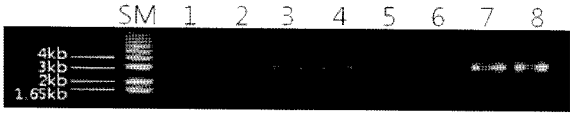

Fig. 3. Agarose gel electrophoresis of plasmids of *Pectobacterium*. Re-isolated plasmids from transformants were treated with *Eco*RI. Lane 1, 2 and 3, 4 showed the plasmids isolated from the white colonies and red colonies of *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed), respectively. Lane 5, 6 and 7, 8 showed the plasmids isolated from the white colonies and red colonies of *P. carotovorum* subsp. *betavasculorum* (pDsRed), respectively.

ing that pGFPuv and pZsYellow would be stably maintained in *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum.*

Effect of antibiotics on the plasmids stability in *Pectobacterium* spp.

To investigate pUC-derived plasmids stability with or without the antibiotic selection pressure, *P. carotovorum* subsp. *carotovorum* (pDsRed) and *P. carotovorum* subsp. *betavasculorum* (pDsRed) were cultured in LB with or without ampicillin. Since rifampicin is a marker for the host bacteria, the effect of rifampicin on the stability of the plasmids in *Pectobacterium* was also tested. The stability of the plasmids was very different depending on the host

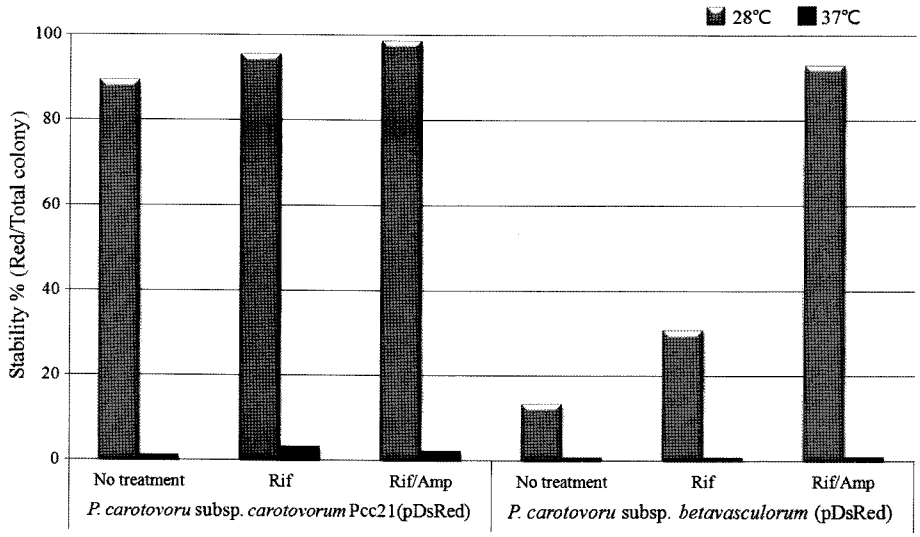

Fig. 4. Stability of pDsRed plasmid in *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum* with various antibiotics at two different temperature conditions.

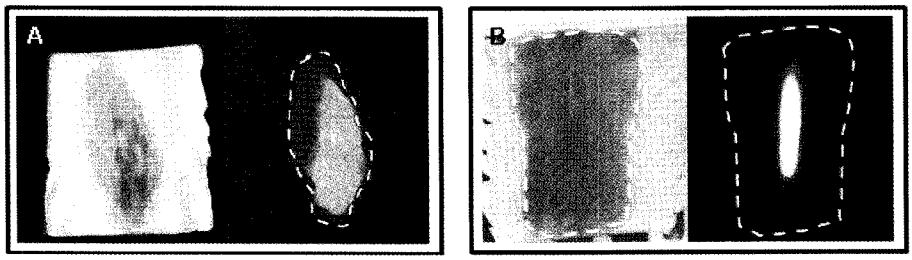

Fig. 5. Fluorescence from the Chinese cabbage infected with *Pectobacterium* marked plasmid. **A**, *P. carotovorum* subsp. *carotovorum* Pcc21 (pGFPuv); **B**, *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed). Dotted lines are the area where the soft rot symptom shows.

bacteria (Fig. 4). As the result, the stability of pDsRed in *P. carotovorum* subsp. *carotovorum* Pcc21 was high. After incubation with ampicillin and rifampicin in media, the stability was 98.56%. Without ampicillin pressure in the media, the stability of pDsRed in *P. carotovorum* subsp. *carotovorum* Pcc21 was still 92.34%. However, the stability of the plasmid pDsRed in *P. carotovorum* subsp. *betavasculorum* was changed dramatically without antibiotic selection pressure. Without any antibiotic pressure, only 13.26% of *P. carotovorum* subsp. *betavasculorum* kept pDsRed compared to 89.22% of *P. carotovorum* subsp. *carotovorum* Pcc21. Thirty one percent of *P. carotovorum* subsp. *betavasculorum* (pDsRed) transformants kept the plasmid pDsRed in the presence of rifampicin antibiotics for the host cell selection. Although the rifampicin is not a selection antibiotics for the plasmid pDsRed, the addition of rifampicin increased the number of *P. carotovorum* subsp. *betavasculorum* (pDsRed) transformants keeping the plasmid pDsRed. Almost all transformants kept the plasmid pDsRed with both ampicilin and rifampicin antibiotics regardless of the host bacteria.

These results indicated that different bacterial strains, even though they belonged to the same species, showed very different sensitivity to the antibiotic pressure in the media. In these experiments, *P. carotovorum* subsp. *betavasculorum* was much more sensitive to the presence of antibiotics in the media than *P. carotovorum* subsp. *carotovorum* Pcc21.

Effect of temperature on the stability of the plasmids in *Pectobacterium* spp.

It was suggested that environmental conditions, which bacterial cells confront, influence plasmid stability (Godwin and Slater, 1979). Since the temperature is one of the most important factors in the bacterial growth, effect of the temperature on the stability of the plasmids in *Pectobacterium* strains was investigated. Optimum temperature for *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *betavasculorum* is 28°C, while they can survive at 37°C. Therefore, effect of the temperature on the stability of the plasmid pDsRed in *Pectobacterium* strains had been conducted at two different temperatures, 28°C and 37°C. Effect of the temperature on the stability of the plasmids in *Pectobacterium* strains was highly significant (Fig. 4). As shown in above experiments, both bacterial strains kept plasmid pDsRed well in the medium containing antibiotics ampicilin and rifampicin at 28°C. When the incubation temperature shifted to 37°C, the ratio of bacterial cells carrying plasmid was dropped dramatically regardless of the bacterial strains. Almost all bacterial strains lost the plasmid pDsRed at the extreme temperature for their growth. These results showed that the temperature change gives much stronger stress for the stability of the plasmid in the host bacteria than the antibiotic pressure.

Stability of the plasmids in *Pectobacterium* spp. in plant tissues

Since the antibiotic pressure was relatively weaker than the stress caused by the temperature change, the *Pectobacterium* strains may keep traceable plasmid pDsRed well in the plant tissues without antibiotics unless the temperature was not changed dramatically to 37°C. Therefore, the stability of those plasmids in *Pectobacterium* strains on the plant tissues was tested. The pathogenicity of all transformants had been confirmed before the experiments. Presence of the plasmid in the bacterial strains did not affect the virulence of the host bacteria. All transformants of *P. carotovorum* subsp. *carotovorum* Pcc21 caused severe soft rot on Chinese cabbage. Since Chinese cabbage is not the host plant for *P. carotovorum* subsp. *betavasculorum*, transformants of *P. carotovorum* subsp. *betavasculorum* did not show severe soft rot on the Chinese cabbage but they showed necrosis on the beet (data not shown).

Stability of the fluorescent plasmids (pGFPuv, pZsYellow, pDsRed) and the expression of the fluorescent proteins in *P. carotovorum* subsp. *carotovorum* Pcc21 strains were found to be stable at 28°C. To investigate the potential of the plasmids as a marker or a tracer for *Pectobacterium*, transformed *P. carotovorum* subsp. *carotovorum* Pcc21 strains were infiltrated into Chinese cabbage and expression of

fluorescens proteins were monitored. Three of transformants showed severe disease symptoms at 1 day after inoculation, however the reaction of the fluorescence was different depending on the type of plasmids in *P. carotovorum* subsp. *carotovorum* Pcc21. In case of pZsYellow, bacterial invaded region induced yellow fluorescence even without any plasmids (data not shown), probably due to plant defense response. It was previously reported that yellow fluorescence is induced from the defense response by infiltration of any bacteria in the plant tissue (Dixon and Paiva, 1995). Therefore, it was not able to distinguish the yellow fluorescence expressed by the presence of pZsYellow. Green fluorescence from *P. carotovorum* carrying pGFPuv was detected well in the whole symptom area of Chinese cabbage. Green fluorescence was detected up to 5 cm away from the inoculated area at 1 day after inoculation. Therefore, green fluorescence protein could be used as a good marker or tracer to monitor pathogenic bacteria in plant tissue. *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed) produced red pigment in plate or in plant that could be observed even with naked eyes; this could be a very good character for red fluorescence protein as a marker or tracer but the region for color development by *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed) was highly restricted. The red fluorescent protein could be only observed at the small inoculation area even though the transformed strain had spread far (Fig. 5). Red fluorescens could be barely observed after more than five days of inoculation. According to the slow expression of red fluorescens from *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed) in plant tissues, the DsRed might need to be further engineered using other plasmid with different origin of replication. To investigate the maintenance of pDsRed in *Pectobacterium* in plant tissue, *P. carotovorum* subsp. *carotovorum* Pcc21 (pDsRed) was re-isolated at the infected tissues. The strains isolated from the infected plant tissues kept the plasmids, however they did not express the fluorescence (data not shown). Since the red color of DsRed in *Pectobacterium* was possible to be seen with naked eyes, plasmid pDsRed was applicable as a marker, but the color change was so slow that additional manipulation to increase the expression speed is necessary.

In this study, we investigated the potential utility as a marking system of three different pUC-derived plasmids with fluorescens marker in *P. carotovorum* subsp. *carotovorum* Pcc21 and *P. carotovorum* subsp. *Betavasculorum*. The pUC origin plasmids in *Pectobacterium* were very stable except pDsRed in *P. carotovorum* subsp. *betavasculorum*. Plasmid pZsYellow has disadvantage due to the natural fluorescence by the defense system of the plant tissue. Plasmid pDsRed could serve as a good marker if it is kept at proper antibiotics-containing media, although the color development seems to be slow. The pDsRed was unstable at high temperature, so it may need caution to use the plasmid at high temperature. It is likely that pGFPuv could be an optimum fluorescent marker for *Pectobacterium* strains for its distinct fluorescens expression and excellent tracing.

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