

## CUCUMOPINE TYPE *AGROBACTERIUM RHIZOGENES* K599 (NCPB2659) T-DNA MEDIATED PLANT TRANSFORMATION AND ITS APPLICATION

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### Abstract

*Agrobacterium rhizogenes* infects plants to induce hairy roots. It is classified into four different types such as agropine, mannopine, cucumopine and mikimopine. *A. rhizogenes* K599, also known as NCPB2659, is a representative strain of the cucumopine type. In this review, we discussed the functions of 11 genes on the T-DNA of Ri plasmid pRi2659 in K599. We summarized the infectivity of K599 on a variety of plants and reviewed the applications of K599-induced hairy roots. These applications include analyses of gene and recombinant plasmid functions, direct employment as root parasite host and model system for the interaction between root and its parasite, production of herbal active ingredients as bioreactor, and the regeneration of transgenic plant. We also prospected the application potentials of *A. rhizogenes* K599 in transgenic plant and the mechanism of K599-mediated transgenic plant.

### Introduction

*Agrobacterium rhizogenes* is a gram-negative soil bacterium that belongs to the genus *Agrobacterium* and family *Rhizobiaceae* with an optimum growth temperature of 28°C (White and Nester 1980). It infects plants to induce hairy roots from the infection site, which is a result of the integration of T-DNA of the Ri plasmid of *A. rhizogenes* into plant genome (Chilton *et al.* 1982; Giri and Narasu 2000). The hairy roots harbor certain morphological characters, which roots are branched, pleiotropic and grow quickly on medium without plant hormones. Besides, hairy roots are maintaining the characteristics of the ordinary root system anatomically, and possesses highly stable hereditary since they originate from single-cells containing no chimeras (Banerjee *et al.* 2012, Peebles *et al.* 2009). According to opines production after infection, *A. rhizogenes* is classified into four different types, namely agropine, mannopine, cucumopine, and mikimopine. The Ri plasmid in the agropine type *A. rhizogenes* has two T-DNA regions TL-DNA (15-20 kb) and TR-DNA region (more than TL-DNA in length) that are linked by non-insertion region. The other three types of T-DNA is one-single continuous region in Ri plasmid containing *rol* genes and opine synthesis genes but no auxin synthesis genes (Combard *et al.* 1987, Kiyokawa *et al.* 1994, Guillon *et al.* 2006).

*Genes and their functions on the T-DNA of Ri plasmid of A. rhizogenes K599:* K599 as a representative strain of cucumopine type *A. rhizogenes*, was isolated from soil by an Australian scholar, Allen Kerr (Savka *et al.* 1990). It contains endogenous Ri plasmid pRi2659 which is 185,462 bp in length with a 14,982 bp T-DNA (GenBank accession number: EU186381). The T-DNA region contains a total of 11 genes/ORFs, which are *orf2*, *orf3*, *orf4*, *orf8*, *rolA*, *rolB*, *rolC*, *rolD* (*orf13*), *rolE* (*orf13a*), *orf14* and *cus*, with *orf4* being nested in *orf3* (Fig. 1) (Mankin *et al.* 2007).

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It has been indicated that *rolA* (*rolα*), *rolB* (*rolβ*), and *rolC* (*rolγ*) genes regulate formation and development of hairy roots induced by K599, and *cus* gene encodes the enzyme for cucumopine synthesis (Failla *et al.* 1990; Serino *et al.* 1994). Many reports have shown that homologous *rolA*, *rolB*, *rolC*, and *rolD* genes in other *A. rhizogenes* are also involved in hair root induction (Casanova *et al.* 2005). Hansen *et al.* (1994) proposed that *rolE* (*orf13a*) in mannopine type *A. rhizogenes* 8196 is a class of regulatory proteins, while Aoki *et al.* (1994) believed that *orf14* assisted *rol* hairy root induction. Otten and Helfer (2001) reported that *orf8* gene in *A. rhizogenes* affected glucose metabolism in plants, while Umber *et al.* (2005) found that *orf8* transgenic tobacco plants appeared shorter and variegated. We cloned *orf3* gene from K599 and determined

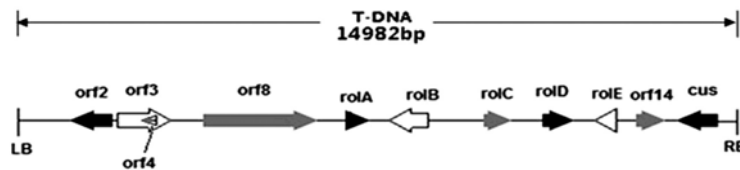


Fig. 1. T-DNA structure of Ri plasmid of cucumopine type *Agrobacterium rhizogenes* K599.

that its open reading frame (ORF) is 1,479 bp encoding a protein of 492 amino acids. The protein sequence deduced from K599 *orf3* is highly homologous to the protein sequence of *orf3* encoded by the *orf3* gene on *A. rhizogenes* Ri plasmid, pRi1724, reported by Moriguchi *et al.* (2001). We investigated the function of *orf3* cloned from K599 by *A. tumefaciens*-mediated transgenic technology and observed decreased internode length and more branching in transgenic tobacco and *Arabidopsis* plants (Wang *et al.* 2016). However, no studies on the functions of *orf2* and *orf4* in K599 have been reported yet. The T-DNA sequences in pRi2659, mikimopine type Ri plasmid pRi1724 (GenBank accession number: AP002086.1, NC\_002575), and agropine type *A. rhizogenes* Ri plasmid pRiA4 (GenBank accession number: K03313, X03433, X51338, X12579, X04833) (Slightom *et al.* 1986) are somewhat similar (Table 1).

**Table 1. Comparison of K599 Ri plasmid pRi2659, mikimopine type MAFF03-01724 plasmid pRi1724, and agropine type A4 plasmid pRiA4.**

| Gene                          | pRi2659 in K599 |                    | pRi1724 in MAFF03-01724                                  |   | pRiA4 in A4  |   |
|-------------------------------|-----------------|--------------------|--|---|--|---|
|                               | ORF length (bp) | No. of amino acids | ORF length (bp)/maximum matching percentage with pRi2659 | No. of amino acids/maximum matching percentage with pRi2659 | ORF length (bp)/maximum matching percentage with pRi2659 | No. of amino acids/maximum matching percentage with pRi2659 |
| <i>orf2</i>                   | 825             | 274                | 825/100%   | 274/100%  | -  | -   |
| <i>orf3</i>                   | 1479            | 492                | 1479/99%   | 492/99%   | 1080/82%   | 359/46.1%   |
| <i>orf4</i>                   | 366             | 121                | 366/99%  | 121/98.3%   | 366/86%  | 121/68.6%   |
| <i>orf8</i>                   | 2343            | 780                | 2286/99%   | 761/83.8%   | 2300/82%   | 766/70.8%   |
| <i>rolA</i>                   | 282             | 93                 | 282/99%  | 93/99%  | 303/73%  | 100/60%   |
| <i>rolB</i>                   | 840             | 279                | 840/98%  | 279/98%   | 780/44.6%  | 259/75%   |
| <i>rolC</i> ( <i>orf12</i> )  | 543             | 180                | 543/99%  | 180/99%   | 543/87%  | 180/74%   |
| <i>rolD</i> ( <i>orf13</i> )  | 597             | 198                | 597/97%  | 198/93%   | 603/91%  | 200/84.8%   |
| <i>rolE</i> ( <i>orf13a</i> ) | 273             | 90                 | 273/99%  | 90/99%  | -  | -   |
| <i>orf14</i>                  | 567             | 188                | 567/98%  | 188/98%   | 552/86%  | 183/75.5%   |
| <i>cus/mis/ags</i>            | 792             | 263                | 987  | 294   | 1191   | 397   |

**Plant hairy root formation induced by *A. rhizogenes* K599:** Many studies have shown that *A. rhizogenes* K599 can efficiently induce hairy root in a wide range of dicotyledonous and monocotyledonous plants such as soybean, carrot, cucumber, impatiens flower, chrysanthemum, corn, petunia, bellflower, *Glycyrrhiza glabra*, cotton, *Artemisia annua*, tomato, and milk vetch etc (Table 2). In particular, K599 is highly infectious on leguminous plants and most of related reports were achieved in soybean and related genera. For example, Cho *et al.* (2000) reported 54 - 95% hairy root induction frequency by K599 in soybean with different genotypes. Xiang *et al.* (2005) used wild-type K599 to infect the cotyledons of soybean, cucumber, and balsam to induce hairy roots at frequencies of 100, 65 and 91%, respectively. K599 also induced hairy root formation in cucumber buds without cutting at a frequency of 10%. The frequency of root formation induced by K599 infection on the engraved aseptic chrysanthemum leaves *in vivo* was 88% (Xiang *et al.* 2011). K599 appeared a suitable progenitor for new *Agrobacterium* strains for plant transformation.

The hairy roots induced by *A. rhizogenes* infection may have different morphology in plant species (Cleene and Ley 1981). Cao (2012) observed the similar phenomenon with K599. The morphology of the adventitious roots induced by K599 on cucumber hypocotyls was divided into two types: type I being branchy and type II being non-branchy with or without woolly roots (Fig. 2A). However, Chrysanthemum (Wang and Xiang 2009) (Fig. 2B) and Petunia (Xu and Xiang 2008; Zhang *et al.* 2011) (Fig. 2C) with the same infection formed typical woolly roots, while soybean only formed non-branched roots (Xiang *et al.* 2005) (Fig. 2D). The morphological variations were seen among the different species in terms of length, thickness and the site of hairy root induction. The results suggest that variations in species, strain and explant type led to different hairy root induction efficiencies (Aarouf *et al.* 2012, Md Setamam *et al.* 2014).

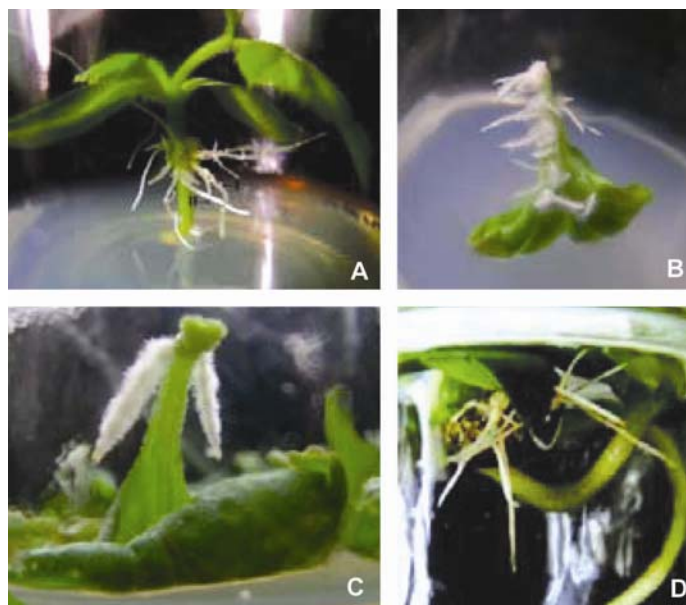


Fig. 2. Different types of hairy roots induced by *Agrobacterium rhizogenes* K599 in different plant species. A: Type I branched hairy root and type II non-branched hairy root on cucumber; B: Woolly hairy roots on the leaf of *Dendranthema morifolium*; C: Woolly hairy roots on the leaf of *Petunia hybrida*; D: Hairy roots without woolly roots on soybean cotyledon.

Table 2. List of plant infected by *A. rhizogenes* K599.

| Plant                                     | Infection site                              | Results  | References  |
|---|---|--|---|
| Soybean<br>( <i>Glycine max</i> )         | cotyledons, apical<br>meristem, hypocotyl   | hairy root or composite plantlet<br>with hairy root              | Savka <i>et al.</i> (1990), Cho <i>et al.</i> (2000), Cho <i>et al.</i> (2004), Collier <i>et al.</i> (2005), Xiang <i>et al.</i> (2005), Kereszt <i>et al.</i> (2007), Yi <i>et al.</i> (2010a), Yi <i>et al.</i> (2010b), Weber and Bodanese-Zanettini (2011), Indrasumunar and Gresshoff (2011), Cao <i>et al.</i> (2011), Li <i>et al.</i> (2011), Guo <i>et al.</i> (2011), Yi <i>et al.</i> (2011), Liang <i>et al.</i> (2013), Dolatabadian <i>et al.</i> (2013) and Xu <i>et al.</i> (2014) |
| <i>Glycine canescens</i>                  | cotyledonary nodes                          | hairy root   | Mohammadi-Deheshmeh <i>et al.</i> (2014)  |
| Carrot ( <i>Daucus carota</i> )           | carrot disc                                 | hairy root   | Serino <i>et al.</i> (1994); Collier <i>et al.</i> (2005)   |
| Sweet potato ( <i>Ipomea batata</i> )     | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| <i>Nicotiana Benthamiana</i>              | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| <i>Nicotiana tabacum</i>                  | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| Potato ( <i>Solanum tuberosum</i> )       | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| Beet ( <i>Beta vulgaris</i> )             | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| <i>Brassica oleracea</i>                  | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| Pumpkin ( <i>Cucurbita sp.</i> )          | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| <i>Datisca glomerata</i>                  | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| <i>Hybiscus esculenta</i>                 | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| <i>Medicago truncatula</i>                | apical stem                                 | composite plantlet with hairy root                               | Collier <i>et al.</i> (2005)  |
| Tomato ( <i>Lycopersicon esculentum</i> ) | leaf disc, direct<br>infection, apical stem | plantlet regeneration from wound<br>leaf                         | Weller and Stead (2002) and Collier <i>et al.</i> (2005)  |
| <i>Petunia hybrida</i>                    | apical stem, leaf                           | plantlet regeneration from hairy<br>root                         | Collier <i>et al.</i> (2005), Xu and Xiang (2008) and Zhang <i>et al.</i> (2011)  |
| <i>Medicago sativa L</i>                  | leaf disc                                   | plantlet regeneration from calli<br>inducing from infection site | Shabnam <i>et al.</i> (1996)  |
| Cucumber ( <i>Cucumis sativus</i> )       | cotyledon, epicotyls,<br>hypocotyl          | composite plantlet with hairy root                               | Xiang <i>et al.</i> (2005) and Meng <i>et al.</i> (2010)  |
| <i>Impatiens balsamina L.</i>             | cotyledon                                   | hairy root   | Xiang <i>et al.</i> (2005)  |

(Contd.)

Table 2 (contd.)

| Plant                                   | Infection site   | Results                               | References  |
|---|--|---------------------------------------|---|
| Chrysanthemum                           | leaf of aseptic seedling   | plantlet regeneration from hairy root | Wang and Xiang (2009) and Xiang <i>et al.</i> (2011)  |
| Corn ( <i>Zea mays</i> )                | wounding plant tissue e.g. leaf, cotyledon, or root hypocotyls                                       | composite plantlet with hairy root    | Runo <i>et al.</i> (2012)                             |
| <i>Elaeagnus angustifolia</i>           | cotyledonary wound   | hairy root                            | Berg <i>et al.</i> (1992)                             |
| Liquorice ( <i>Glycyrrhiza glabra</i> ) | cotyledon, hypocotyl, leaf   | hairy root                            | Chen and Hou (1991) and Mehrotra <i>et al.</i> (2008) |
| Cotton ( <i>Gossypium hirsutum</i> )    | cotyledon  | hairy root                            | Zhang (2011)  |
| <i>Podophyllum hexandrum</i>            | embryo   | hairy root                            | Giri <i>et al.</i> (2001a)                            |
| <i>Artemisia annua</i>                  | shoot-tip meristem   | hairy root                            | Giri <i>et al.</i> (2001b)                            |
| Grapevines ( <i>Vitis spp.</i> )        | internode, stem-cut, petiole-cut surfaces on in vitro plant, young shoot excised from in vitro plant | hairy root                            | Jittayasothorn <i>et al.</i> (2011)                   |
| Peanut ( <i>Arachis pintoi</i> )        | hypocotyl  | hairy root                            | Shen <i>et al.</i> (2012)                             |
| <i>Astragalus sinicus</i>               | hypocotyl  | composite plantlet with hairy root    | Jia <i>et al.</i> (2012)                              |
| <i>Tetrastigma hemsleyanum</i>          | leaf   | hairy root                            | Du <i>et al.</i> (2015)                               |
| <i>Phaseolus spp</i>                    | wounding the cotyledonary node with a syringe  | hairy root                            | Estrada-Navarrete <i>et al.</i> (2006)                |
| <i>Lotus corniculatus</i>               | stem section with one node and root  | plantlet regeneration from hairy root | Jian <i>et al.</i> (2009)                             |
| Strawberry                              | leaf, petiole, and terminal bud  | hairy root, transgenic plant          | Tavizi <i>et al.</i> (2015)                           |

*Application of hairy root induced by A. rhizogenes K599:* The hairy roots have important applications. Kereszt *et al.* (2007), Estrada-Navarrete *et al.* (2006) and our group used *A. rhizogenes* K599 to infect soybean, bean, and cucumber to successfully generate composite plantlets with hairy roots (Fig. 3A) and studied root-related genes. Using transformed hairy roots harboring different function genes on the composite transgenic soybeans, Cao *et al.* (2011) found that overexpression of *TaNHX2* improved the plant salt-tolerance; Guo *et al.* (2011) analyzed the correlation between the *GmEXPB2* and soybean root development; Liang *et al.* (2013) discovered that the soybean malic acid transporter *GmALMT1* regulated malate exudation through *GmALMT1* to improve soybean adaptation to acid soils; Dolatabadian *et al.* (2013) investigated the ability of *GmNFR5a* to improve the ability of soybean resistance to salt stress. Using the hairy roots of transgenic soybeans, Yi *et al.* (2010a, 2010b) investigated the functions of the *chs* gene family in soybean; Indrasumunar *et al.* (2011) studied the function of lipo-oligochitin-like receptor. Lin *et al.* (2011) constructed the pHairyRed plasmid containing the red fluorescent protein gene, while Alzohairy *et al.* (2013) constructed the pJan25 plasmid containing the green fluorescent protein. Both plasmids were transferred into the wild-type K599 for the infection of soybean explants to obtain transgenic hairy roots, which verified the effectiveness of the recombinant plasmid. Xu and Xiang (2008) constructed a plasmid pBIN-35S-GFP with green fluorescent protein gene, and obtained highly expressed GFP in transgenic hairy roots of petunia that were infected with K599 containing pBIN-35S-GFP (Fig. 3B). Hairy root is an efficient tool for functional analysis of genes. Moreover, it is well adapted to RNAi-based approaches to study gene function, particularly in case of abiotic and biotic stresses adaptation. Notably, leguminous plant (e.g. soybean) is known to be a recalcitrant species to plantlet regeneration of genetic transformation via *Agrobacterium tumefaciens*, while *A. rhizogene* K599-mediated transformation offers a rapid alternative to study soybean gene functions in hairy root.

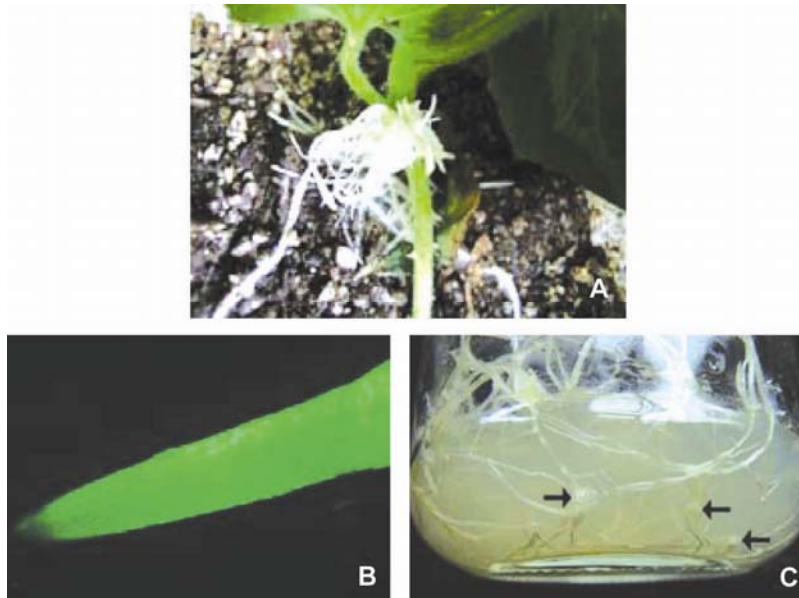


Fig. 3. The application of hairy roots. A: The composite cucumber plantlet with hairy root. B: Hairy root with *gfp* gene emitted strong green fluorescence under fluorescent microscope. C: *Meloidogyne incognita* was propagated in cucumber hairy root and the root knot was observed (arrow).

Both Savka *et al.* (1990) and Cho *et al.* (2000) successfully propagated soybean cyst nematodes using the hairy roots induced by K599 infection of soybean cotyledons. Runo *et al.* (2012) used composite corn plant containing hairy roots to investigate the interaction between corn and its parasitic plant *Striga hermonthica*. We also successfully cultured root knot nematodes using the hairy roots induced by K599 infection of cucumber cotyledons (Fig. 3C). Valuably, the hairy roots that express the gene of interest can be obtained reproducibly and rapidly, and can be used for identification the gene resistance to nematode.

Giri *et al.* (2011a, 2011b) reported that the hairy roots induced by K599 infection were able to effectively produce pureonebio and artemisinin. Yi *et al.* (2010, 2011) discovered that over-expression of *CHS8*, *IFS2* or *CHS8 + IFS2* in K599-induced hairy roots led to increased contents of root isoflavone. Du *et al.* (2015) used K599 to infect the medicinal plant *Tetradigma hemsleyanum*, which resulted in higher contents of total flavonoids in the hairy roots. Especially, the greatest advantage of hairy roots is that they often exhibit similar or greater biosynthetic capacity for secondary metabolite production as compared to their mother plants. Hairy root cultures are also known to produce a spectrum of secondary metabolites that are not present in the parent plant (Srivastava *et al.* 2007, Chandra 2012).

Furthermore, hairy roots are often able to regenerate whole viable plants and maintain their genetic stability during continuous subculturing and plant regeneration. Jian *et al.* (2009) regenerated the whole plants from *Lotus corniculatus* hairy roots induced by K599. Zhang *et al.* (2011) induced hairy roots after K599 infection of petunia leaf explants and regenerated *cyp2e1* transgenic plants. In addition, the hairy roots induced by K599 infection of *chrysanthemum* leaves formed calli, which were then regenerated into the whole plants; the whole plants showed characteristics of dwarf and more roots with normal flowering (Xiang *et al.* 2011).

### **Perspectives of *A. rhizogenes* K599 in the application**

*A. rhizogenes* K599 has a high infectivity on a variety of plants, especially on early recalcitrant leguminous plants. K599-induced hairy roots have many applications. As mentioned above, transgenic hairy roots can be directly used for studies on genes, plasmid functions and those on the interactions between plants and their root pests; they can be used as bioreactors for the production of active ingredients of medicinal plants, and they may also be used to regenerate complete transgenic plants. In the future, the hairy roots induced by K599 will show the most promising application in root metabolic engineering and producing valuable medicine ingredients.

Ri plasmid pRi2659 in K599 carries a single consecutive T-DNA region containing only 11 genes/coding regions. Because of its simple form, it can serve as the model *A. rhizogenes* to probe process of the insertion and integration of T-DNA (Wang *et al.* 2016). Although the whole sequence of plasmid pRi2659 in K599 has been determined, the functions of the majority of the coding regions on the plasmid, including the *orf2* and *orf4* genes on T-DNA, have not been revealed. Whether *rolE* (*orf13a*), *orf14* and *orf8* are required in hairy root induction and the mechanisms need further investigation. In addition, previous studies on the functions of *orf8*, *rolD*, *rolE* and *orf14* have almost cloned them from agropine type or mannopine *A. rhizogenes*, but their homologous genes on K599 T-DNA are somewhat different. Do the differences lead to its role in higher invasion on plants than other strains? Remarkably, the draft genome sequence of strain K599 has been reported recently (Valdes Franco *et al.* 2016). However, it is poorly understanding which genes in the genome are relation with infectivity to plant cell. Therefore, improved functional studies on related genes should contribute significantly to the in-depth understanding of the infection mechanism of K599.

On the other hand, *A. rhizogenes* K599 has strong infectivity on some dicotyledons, especially cucumber and legumes, and certain infectivity on monocots like corn. However, it is

unknown whether K599 infects rice, wheat, and other important crop plants. Although K599 infection-induced hairy roots of transgenic petunia, *Lotus corniculatus*, and chrysanthemum have been successfully used to regenerate transgenic plants, no success has been reported on the regeneration from hairy roots of soybean and other monocotyledonous crops. Further studies on such topics should help explore the potential applications of K599 in transgenic plants.

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