Chapter 12
Hairy Roots as a Tool for the Functional Analysis of Plant Genes

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Abstract With its root-inducing (Ri) plasmid, *Agrobacterium rhizogenes* is a valuable alternative to transfer gene constructs into the genome of plant species which are difficult to stably transform with disarmed strains of *Agrobacterium tumefaciens*. Composite plants consisting of transformed hairy roots induced on a non-transgenic shoot have been reported in an increasing number of legume and nonlegume plant species. They were first used in the model legumes *Medicago truncatula* and *Lotus japonicus* to study the symbiotic interaction with rhizobia. Since then, composite plants have been shown to be effective to investigate the function of genes involved in mycorrhizal symbiosis, root-nematode and root-pathogen interactions, resistance response of plant roots to parasitic weeds, root development and branching, and the formation of wood. The different methodologies developed to generate composite plants and the applications of co-transformed hairy roots for studying gene function are discussed in this chapter, together with recent opportunities offered by genome editing technologies in hairy roots.

Keywords *Agrobacterium rhizogenes* · Composite plant · Gene functional analysis · Genome editing · Hairy root

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12.1 Introduction

Once a gene has been isolated, the exploration of its function begins with DNA sequence analysis together with a search of public databases for characterized genes with similar sequences. However, such a comparison involves certain risks since similarity at the nucleotide level does not always mean the gene product will have a similar structure and function. Additional information can be obtained by analyzing the spatiotemporal expression of the studied gene and its response to several biotic and abiotic factors. Nevertheless, one of the most significant steps in the functional analysis usually involves the study of transgenic plants in which the gene has been knocked out by mutagenesis, overexpressed, or downregulated (Rhee and Mutwil 2014). With the alterations observed in the plant phenotype, important conclusions can be drawn concerning the function of the corresponding gene.

Agrobacterium tumefaciens-mediated transformation is the most popular technique to generate transgenic plants. However, a major problem linked to the use of this bacterium is the need for efficient organ regeneration and transformation in plants (Anami et al. 2013). To study genes expressed in plant roots, *Agrobacterium rhizogenes* offers a valuable alternative to disarmed strains of *A. tumefaciens*. This gram-negative soil bacterium is responsible for the development of hairy root disease in many dicotyledonous plants as well as in some gymnosperms and monocotyledonous plants (Tepfer 1990). In a process similar to that described for *A. tumefaciens*, *A. rhizogenes* transfer into the genome of the infected host plant a T-DNA fragment from the bacterial root-inducing (Ri) plasmid carrying oncogenes that encode enzymes which control auxin and cytokinin biosynthesis (Koplow et al. 1984; Britton et al. 2008). The resulting modifications in the hormonal balance induce the formation of roots at the wounding site which are morphologically different from normal roots. The so-called hairy roots are characterized by rapid hormone-independent growth, are much more branched, have numerous root hairs, and exhibit plagiotropic root development. *A. rhizogenes* has proven to be a valuable tool for generating transgenic roots which are easy to grow and can be used for a range of biological applications including metabolic engineering and phytoremediation, as well as for the production of valuable secondary metabolites and recombinant proteins (Guillon et al. 2006; Talano et al. 2012; Mehrotra et al. 2015).

A. rhizogenes hairy roots have other valuable applications in many areas of basic plant research. This pathogenic bacterium can be used to generate composite plants consisting of transformed hairy roots induced on a non-transgenic shoot (Beach and Gresshoff 1988; Hansen et al. 1989; Collier et al. 2005). Binary vectors carrying appropriate gene constructs can be introduced into oncogenic strains of *A. rhizogenes*; the resulting bacteria can then be used to obtain co-transformed hairy roots which integrate both the T-DNA from the Ri plasmid and the T-DNA from the genetically engineered binary vector. The co-transformation procedure enables more rapid analysis of transformed roots than the methods used to generate plants which are stably transformed by disarmed *A. tumefaciens* or by direct gene

Table 12.1 Composite plant-inducible families and species

Family	Genus/species
Apiaceae	*Daucus carota*
Brassicaceae	*Arabidopsis thaliana* *Brassica oleracea*
Casuarinaceae	*Allocasuarina verticillata* *Casuarina glauca*
Chenopodiaceae	*Beta vulgaris*
Convolvulaceae	*Ipomoea batatas*
Cucurbitaceae	*Cucurbita pepo*
Datiscaceae	*Datisca glomerata*
Fabaceae	*Aeschynomene indica* *Arachis hypogaea* *Glycine max* *Lotus corniculatus* *Lotus japonicus* *Lupinus albus* *Medicago truncatula* *Phaseolus vulgaris* *Pisum sativum* *Sesbania rostrata* *Trifolium pratense* *Trifolium rubens* *Vicia hirsuta* *Vigna aconitifolia* *Vigna unguiculata*
Lauraceae	*Persea americana*
Malvaceae	*Hibiscus esculenta*
Myrtaceae	*Eucalyptus camaldulensis* *Eucalyptus grandis*
Poaceae	*Zea mays*
Rhamnaceae	*Discaria trinervis*
Rosaceae	*Prunus* spp.
Rubiaceae	*Coffea arabica*
Salicaceae	*Populus* sp.
Solanaceae	*Lycopersicon esculentum* *Nicotiana benthamiana* *Nicotiana tabacum* *Petunia x hybrida* *Solanum tuberosum*
Theaceae	*Camellia sinensis*

Adapted from Collier et al. (2005) and completed with recent references

transfer techniques, such as biolistic or protoplast electroporation. Composite plants have now been reported in at least 18 plant families including about 40 species (Table 12.1), and the utility of the co-transformed hairy roots for investigating the function of genes involved in different aspects of root development and biotic interactions is now well established.

Different methods to generate composite plants using *A. rhizogenes* are described in this chapter, and their contribution to the functional analysis of candidate genes involved in different physiological processes is illustrated. In addition to promoter studies and downregulation of gene expression resulting from RNA interference (RNAi) experiments, the recent development of genomic mutations induced by the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9) system in composite plants is presented.

12.2 *Agrobacterium Rhizogenes*-Mediated Transformation: Technical Aspects

12.2.1 Factors Involved in a Successful T-DNA Transfer by A. rhizogenes

A successful transfer of a T-DNA into a plant species involves many factors that need to be optimized, especially when the host is poorly susceptible to agrobacterial infection. Fortunately, knowledge of the molecular mechanisms underlying the interaction between plants and agrobacteria has progressed remarkably in recent years, making it possible to genetically transform an increasing number of plant species (Lacroix and Citovsky 2013). One of the first factors to consider is the choice of the *A. rhizogenes* strain. Several strains need to be tested to identify the bacteria capable of inducing hairy roots with a phenotype that is as close as possible to the one of the non-transformed roots. A very pronounced hairy root phenotype can profoundly alter root architecture and biotic interactions, thus affecting conclusions drawn concerning the function of the candidate genes (Dolatabadian et al. 2013). In the actinorhizal shrub *Discaria trinervis*, for example, both A4RS and ARqua1 strains induced transgenic roots at the site of infection, but they differed in their phenotype (Imanishi et al. 2011). Since the hypervirulent strain A4RS had a strong impact on root architecture, further experiments with composite plants of *D. trinervis* were conducted with Arqua1. The second factor involved in a successful gene transfer is indeed linked to the host, which needs to provide the appropriate signaling molecules to activate the virulence genes of *A. rhizogenes*. The addition of exogenous phenolic compounds such as acetosyringone can sometimes improve the dialogue between the host and the agrobacterium (Lacroix and Citovsky 2013).

The co-cultivation phase, during which the host and the bacteria are usually in contact for 1–6 days, is another critical step in the interaction. It has been shown that the T-DNA transfer and integration can be affected by bacterial density, plant age and growth conditions, light, gas exchange, nutrient medium, growth regulators, pH, and humidity, among other factors (Karami 2008). Unfortunately, optimal conditions for the genetic transformation have to be studied empirically, requiring intensive work to combine the different factors.

12.2.2 In Vitro and Ex Vitro Transformation Procedures

Both in vitro and ex vitro techniques have been successfully used to generate composite plants. In the in vitro process, plants have to be germinated, grown, and manipulated in aseptic conditions. When young seedlings are used, inoculations with *A. rhizogenes* can be achieved by wounding the hypocotyls with a needle dipped in a fresh colony (or culture in exponential growth) of the chosen *A. rhizogenes* strain (i.e., Diouf et al. 1995). It has also been reported that the agrobacterium culture can be injected directly into the stem (Markmann et al. 2008). An alternative procedure consists in sectioning the radicle of the seedlings with a scalpel and then coating the sectioned surface with *A. rhizogenes* (Boisson-Dernier et al. 2001). The rate of development of hairy roots varies considerably depending on the method of infection. For instance, in the tropical tree *Casuarina glauca*, hairy roots developed in less than 30% of the plants with a sectioned hypocotyl, whereas when hypocotyls were inoculated with a needle, hairy roots developed on 95% of the plants (Svistoonoff et al. 2010). To obtain the composite plants, the normal non-transformed root system is removed about 3 weeks after inoculation with *A. rhizogenes*, and when possible, co-transformed roots containing the newly introduced genes from the appropriate binary vector are selected. It should be noted that, even though the shoot is not transgenic, composite plants sometimes exhibit an altered aerial part with shorter internodes. This alteration of phenotype is probably linked to the modification of the hormonal balance in the transgenic root system displaying the hairy root phenotype.

Ex vitro procedures may be preferred to avoid the constraints and costs linked to tissue culture and aseptic conditions. This technique was first reported in 2005 with the introduction and expression of the reporter gene *gfp* in hairy roots induced in 14 different plant species belonging to five different orders including nine plant families (Collier et al. 2005). Apical stems from young plants were directly inserted into rockwool cubes containing the *A. rhizogenes* inoculum. After 3 weeks to 2 months, hairy roots were observed on 56–100% of the inoculated stems, depending on the plant species. The major challenge of this simple procedure is preventing the dispersal of the transgenic pathogenic rhizobacteria in the environment, thus requiring an appropriate confined growth chamber or glasshouse.

12.2.3 Selection of Co-transformed Hairy Roots

One advantage of *A. rhizogenes*-mediated transformation is that transgenic roots can be obtained without using a selection agent. Hairy root morphology is used for the primary selection of transgenic roots. However, when performing co-transformation with an *A. rhizogenes* strain harboring a genetically engineered gene construct in a binary vector, a selection procedure with either a reporter gene or an antibiotic resistance gene is usually required to detect the co-transformed roots and to facilitate molecular and phenotypic analyses of the composite plants.

Based on experience gained with the actinorhizal tree *C. glauca*, and the analysis of several hundred composite plants, the rate of co-transformed hairy roots can vary from 20% to 65%. In order to identify the co-transformed roots, a constitutively expressed reporter gene such as ß-glucuronidase (GUS) (Jefferson et al. 1987), DsRED1, or green fluorescent protein (GFP) gene (Haseloff and Siemering 2006) was included in the T-DNA of the binary vector. Reporter genes encoding fluorescent proteins appeared to be the best candidates since their gene products could be visualized in roots under UV light without affecting the viability of plant tissues. Interestingly, the intensity of fluorescence was generally correlated with the level of expression of the other genes stacked on the T-DNA of the binary vector. In RNAi experiments designed to downregulate symbiotic genes in *C. glauca*, hairy roots displaying a high level of fluorescence also exhibited a strong extinction of the candidate symbiotic gene, as determined by q-RTPCR (Gherbi et al. 2008a).

Kanamycin selection of co-transformed roots has occasionally been performed using the *nptII* gene in the transferred T-DNA. A range of kanamycin concentrations has to be tested to inhibit the growth of non-co-transformed hairy roots on the agar plates. After 2 or 3 weeks of incubation with kanamycin, the non-co-transformed roots stop growing, turn brown, and do not penetrate the agar nutrient medium, whereas the co-transformed roots continue to grow rapidly on the agar. Once the hairy roots have developed, the antibiotic has to be rapidly removed to avoid a negative impact on the growth of the non-transformed aerial part of the composite plants.

12.3 Functional Analyses of Plant Genes in Composite Plants

In recent years, a wide diversity of composite plants have been used to improve the functional analysis of plant genes expressed in roots, the largest number of publications being in the area of plant-microbe interactions (Table 12.2). As illustrated below, the use of *A. rhizogenes* together with that of RNA interference (RNAi) has proven to be very useful to study gene function using reverse genetics.

12.3.1 Study of Interactions Between the Host Plant and Nitrogen-Fixing Microorganisms

Due to the difficulty in obtaining transgenic legumes using *A. tumefaciens*, composite plants were rapidly used to characterize the plant genes involved in the symbiotic process with nitrogen-fixing rhizobia. *A. rhizogenes* transformation was first described for *Lotus corniculatus* (Jensen et al. 1986) and subsequently used in the two model plants *Medicago truncatula* (Boisson-Dernier et al. 2001) and *Lotus*

Table 12.2 Examples of gene function analysis using composite plants obtained after genetic transformation using *Agrobacterium rhizogenes*

Plant	Gene	Function	References
Aeschynomene indica	*gus*	Reporter gene	Bonaldi et al. (2010)
Allocasuarina verticillata	*gus*	Reporter gene	Gherbi et al. (2008b)
Arabidopsis thaliana	*KOJAK* *gfp*	Root hair development Reporter gene	Limpens et al. (2004) Collier et al. (2005)
Arachis hypogaea	*gfp, gus* *Cry8Ea1* *AdEXLB8* *EXLB*	Reporter genes Toxicity against white grubs Nematode resistance Stress-responsive expansin gene	Sinharoy et al. (2009) Geng et al. (2013) Guimaraes et al. (2017a) Guimaraes et al. (2017b)
Camellia sinensis	*gus*	Reporter gene	Alagarsamy et al. (2018)
Casuarina glauca	*gus* *SYMRK* *CHS* *CCaMK*	Reporter gene Root nodulation Flavonoid pathway Root nodulation	Diouf et al. (1995) Gherbi et al. (2008a) Abdel-Lateif et al. (2013) Svistoonoff et al. (2013)
Coffea arabica	*gus*	Reporter gene	Alpizar et al. (2006)
Cucurbita pepo	*DR5-gus, DR5-gfp*	Reporter genes driven by an auxin-responsive promoter	Ilina et al. (2012)
Discaria trinervis	*ENOD11-gus*	Marker of *Frankia* infection	Imanishi et al. (2011)
Eucalyptus camaldulensis	*gfp*	Reporter gene	Balasubramanian et al. (2011)
Eucalyptus grandis	*CCR1*	Lignin biosynthesis	Plasencia et al. (2016)
Glycine max	*IFS* *TIP1* *EXPB2* *SPX3* *Fib-1* *Y25C1A.5*	Isoflavone synthase Salt tolerance Cell wall ß-expansin Phosphorous signaling pathway Nematode reproduction and fitness	Subramanian et al. (2004, 2006), White et al. (2015) An et al. (2017) Guo et al. (2011), Li et al. (2015) Yao et al. (2014) Li et al. (2010)
Lotus japonicus	*gus, luc*	Reporter genes	Stiller et al. (1997)
Lupinus albus	*LaMATE*	Phosphorous stress	Uhde-Stone et al. (2005)
Lycopersicon esculentum	*gfp*	Reporter gene	Collier et al. (2005)
Medicago truncatula	*ENOD11* *ENOD8* *ROP9* *RbohE* *ABCG10* *RDN*	Root infection by *Rhizobium* Root nodulation Oomycete colonization Role in arbuscule development ABC transporter of the G subfamily Root nodulation	Boisson-Dernier et al. (2001, 2005) Coque et al. (2008) Kiirika et al. (2012) Belmondo et al. (2016) Banasiak et al. (2013) Kassaw et al. (2017)
Populus spp.	*YFP*	Reporter gene	Neb et al. (2017)

(continued)

Table 12.2 (continued)

Plant	Gene	Function	References
Persea americana	*gus, gfp*	Reporter genes	Prabhu et al. (2017)
Phaseolus vulgaris	*NIN* *gus, gfp*	Root nodulation Reporter genes	Nanjareddy et al. (2017) Colpaert et al. (2008)
Pisum sativum	*LYK9*	Control of plant immunity	Leppyanen et al. (2017)
Prunus cerasifera	*Ma*	Nematode resistance	Claverie et al. (2011)
Prunus spp.	*Egfp*	Reporter gene	Bosselut et al. (2011)
Solanum tuberosum	*gus*	Reporter gene	Horn et al. (2014)
Vicia hirsuta	*gus*	Reporter gene	Quandt et al. (1993)
Vigna unguiculata	*RSG3-301*	Resistance to *Striga gesnerioides*	Mellor et al. (2012)
Zea mays	*gfp*	Reporter gene	Runo et al. (2012)

In this table, functional analyses include promoter studies, overexpression of the candidate genes, or RNAi experiments to downregulate the genes studied. Reporter genes were usually used to establish the proof of concept of the technology.
ABCG, ATP-binding cassette transporter of the G family; ABC transporter; CCaMK, calcium/calmodulin-dependent kinase; CCR1, cinnamoyl-CoA reductase 1; CHS, chalcone synthase; CRY8Ea1, CRY protein from *Bacillus thuringiensis*; DR5, auxin-responsive promoter; ENOD8, nodule-specific esterase; ENOD11, early nodulin; EXL, expansin-like protein; EXP, expansin gene; gfp, green fluorescent protein gene; gus, ß-glucuronidase gene; IFS, isoflavone synthase gene; KOJAK, cellulose synthase-like protein; LaMATE, *Lupinus albus* multidrug and toxin efflux; luc, luciferase; LYK9, Lys-M-receptor like kinase; Ma, R protein; NIN, nodule inception gene; RbohE, NADPH oxidase; RDN, root-determined nodulation protein; ROP9, GTPase; SPX, protein related to phosphate homeostasis and signaling; SYMRK, symbiosis receptor kinase; TIP, tonoplast intrinsic protein; Yfp, yellow fluorescent protein gene

japonicus (Stiller et al. 1997). Composite plants have also been reported in *Glycine max* (Kereszt et al. 2007; Cao et al. 2009), *Vicia hirsuta* (Quandt et al. 1993), *Vigna aconitifolia* (Lee et al. 1993), *Phaseolus vulgaris* (Estrada-Navarrete et al. 2006; Colpaert et al. 2008), *Trifolium rubens* (Diaz et al. 1989), and *T. pratense* (Diaz et al. 2000).

Composite plants have largely contributed to a better understanding of the symbiotic dialogue established between the host and nitrogen-fixing rhizobial strains, in legumes which develop determinate or indeterminate nodules, and which undergo either an intracellular or intercellular infection process. Whereas mutants in the model plants *M. truncatula* and *L. japonicus* led to the identification of the so-called common symbiotic pathway (CSP) (Gueurts et al. 2016), RNAi experiments in composite plants confirmed that the CSP was also involved in the nodulation process of legumes in which the infection process does not proceed via root hair infection, such as *Sesbania rostrata* (Van de Velde et al. 2003), *Arachis hypogea* (Sinharoy et al. 2009), and *Aeschynomene indica* (Bonaldi et al. 2010).

Major advances have also been made in actinorhizal plants which develop nitrogen-fixing nodules following interaction with the gram-positive actinobacteria

Frankia. Since it takes about 12 months to obtain transgenic nodulated plants of *C. glauca* resulting from a T-DNA transfer by *A. tumefaciens* (Smouni et al. 2002), composite plants were used to generate data on a large number of co-transformed hairy roots more rapidly. This method was first used in 1995 (Diouf et al. 1995) to demonstrate that a promoter from a legume hemoglobin gene kept its spatiotemporal pattern of expression in an actinorhizal nodule, thus suggesting the conservation of molecular mechanisms underlying the nodulation process between actinorhizal plants and legumes. With the development of the RNAi technology, downregulation of two genes isolated from *C. glauca* and sharing homology with the receptor-like kinase SYMRK and the calcium- and calmodulin-dependent kinase CCaMK genes from the CSP in legumes revealed that this pathway was also required by *Frankia* for root infection and nodulation (Gherbi et al. 2008a; Svistoonoff et al. 2013). Additional data obtained in composite plants of two other actinorhizal plants *D. trinervis* (Imanishi et al. 2011) and *Datisca glomerata* (Markmann et al. 2008), which cannot be transformed by *A. tumefaciens*, have also considerably enriched our knowledge of the original nodulation process resulting from *Frankia* intercellular infection.

12.3.2 Plant Mycorrhizal Interactions

Arbuscular mycorrhiza is a major widespread mutualistic association that concerns 80% of land plants and involves fungi of the phylum Glomeromycota. The plant provides carbohydrates to the fungus which, in return, supplies the host with mineral nutrients, especially phosphate, and improves water absorption and disease resistance (Lanfranco et al. 2016).

In *M. truncatula*, together with the possibility to obtain nitrogen-fixing nodules on composite plants, it has been shown that these roots can be colonized by endomycorrhizal fungi, even when the hairy roots are excised from the composite plants and propagated as independent organs (Boisson-Dernier et al. 2001, 2005; Mrosk et al. 2009). With the actinorhizal plant *C. glauca*, it was not possible to grow hairy roots independently, but mycorrhization by *Rhizophagus irregularis* occurred on the hairy roots of composite plants (Gherbi et al. 2008a). Composite legume and actinorhizal plants were further used to compare gene expression during the symbiotic process with rhizobium and/or *Frankia* and in endomycorrhizal associations. These experiments, together with the study of legume mutants, have provided evidence that the common signaling pathway involved in the nodulation process is necessary for all root endosymbioses involving rhizobium, *Frankia*, and AM fungi (Gherbi et al. 2008a; Markmann et al. 2008).

Composite plants have also been used to characterize some candidate genes potentially involved in specific stages of the endomycorrhization process. For example, co-transformed hairy roots of *M. truncatula* highlighted the role of a NADPH oxidase encoded by the gene *RbohE* during arbuscule accommodation within cortical root cells (Belmondo et al. 2016).

12.3.3 Plant Nematode Interaction

Meloidogyne species of root-knot nematodes (RKN) attack the roots of most vegetable, fruit, and ornamental crops under Mediterranean and tropical climates. Infested roots become distorted and develop rounded or irregular galls which alter water and nutrient uptake, thereby reducing plant growth and yield (Fosu-Nyarko and Jones 2016). Composite plants for studying nematode resistance have been documented in *Lycopersicon esculentum* cv. (Collier et al. 2005), *Glycine max* (Li et al. 2010), *Prunus* spp. (Claverie et al. 2011), *Arachis hypogaea* (Guimaraes et al. 2017a), and *Persea americana* (Prabhu et al. 2017).

In *Prunus* species, composite plants have been used to validate the function of the candidate gene *Ma* isolated in *Prunus cerasifera* (Claverie et al. 2011). When co-transformed roots expressed the *Ma* genomic sequence under the control of its native promoter, a high level of resistance was obtained to the three major RKNs *Meloidogyne incognita*, *M. arenaria*, and *M. javanica*.

Several RKN species are pathogenic on *A. hypogaea* and cause considerable yield losses in Africa every year. In wild-type *Arachis* species which are resistant to a number of pests and diseases, transcriptomic studies have identified candidate genes that could contribute to resistance to *M. arenaria*. Since peanut is recalcitrant to genetic transformation, *A. rhizogenes* was tested as an alternative to develop the functional analysis of plant genes. Using the *A. rhizogenes* strain K599, the candidate gene for nematode resistance *AdEXLB8* was overexpressed in hairy roots induced on the peanut cultivar "Runner." Two months after *M. arenaria* infection, a reduction of 98% in the number of galls and egg masses was observed compared to the control hairy roots (Guimaraes et al. 2017b).

12.3.4 Interactions of Hairy Roots with Parasitic Plants

Striga is one of the most important genera of parasitic plants and causes devastating losses in cereal yields in sub-Saharan Africa. It is an obligate hemiparasitic parasite that attaches to host roots, forms a haustorium, and penetrates the root cortex of potential hosts. It then damages cereal crops by draining off water and nutrients, impairing photosynthesis, and having a phytotoxic effect (Yoshida et al. 2016). The combination of these factors severely reduces the growth of the crops and causes the subsequent failure to set seeds. Understanding the molecular mechanisms underlying the plant-parasitic weed interaction is essential for the identification of genes that could improve crop yield via biotechnological or marker-assisted breeding strategies.

The possibility for *Striga* to parasite hairy roots of composite plants has been demonstrated. *Striga gesnerioides* (L.) is a major parasite of the grain legume cowpea (*Vigna unguiculata*) in Africa. Following infection with the *A. rhizogenes* strain R1000, composite plants of *V. unguiculata* were obtained using an ex vitro

protocol, and co-transformed roots were selected using *gfp* as biomarker (Mellor et al. 2012). Up to 80% of the inoculated plants developed at least one transgenic root. When subjected to *Striga*, hairy roots of composite plants responded similarly to wild-type roots of the susceptible cowpea cultivar, allowing the formation and growth of parasite tubercles on the legume transformed roots. When the gene *RSG3-301* encoding a resistance (R) gene to *Striga* was co-transformed in the hairy roots of a susceptible cowpea genotype, its expression resulted in the acquisition of a resistant phenotype. These data demonstrate that the expression of the oncogenes from *A. rhizogenes* has no impact on the cowpea-*Striga* interaction.

Runo et al. (2012) reported a similar approach using *Zea mays*. Using the strain K599, composite maize plants with co-transformed roots were obtained in vitro on 85.3% of the inoculated seedlings. Two weeks after inoculation with *Striga hermonthica*, the number and size of *S. hermonthica* individuals infecting transformed or wild-type roots of maize were identical. Microscopic examination of the infected roots further confirmed that the timing and characteristics of the infection process were not altered in the hairy roots. These data confirm that composite plants will be suitable for the characterization of plant genes which play a critical role in parasitism or host defense.

12.3.5 Hairy Roots for the Study of Wood Formation

Since the regeneration of transgenic forest trees is limited to a small number of species due to poor regeneration ability and difficulty to achieve T-DNA transfer by *A. tumefaciens*, *A. rhizogenes* appeared to be a viable alternative. Composite plants have now been reported in several forest trees including *Eucalyptus camaldulensis* (Balasubramanian et al. 2011) and *E. grandis* (Plasencia et al. 2016) and recently in poplar (Neb et al. 2017).

Whereas poplar has been the main forest tree used to advance our knowledge of the lignification process in forest trees, other trees such as eucalyptus are of major economic value. With the release of the *Eucalyptus grandis* genome sequence, many candidate genes involved in wood formation have been identified, paving the way for functional analysis. Due to the recalcitrance of *E. grandis* to *Agrobacterium*, the hypervirulent A4RS strain had to be used to obtain efficient transformation (62% on average) (Plasencia et al. 2016). Microscopic examination showed that xylem development was similar in both hairy and wild-type roots. A proof of concept of the composite plant approach was obtained with the downregulation of the cinnamoyl-CoA reductase1 gene (*EgCCR1*) in *E. grandis*, encoding a key enzyme from the lignin biosynthetic pathway. As expected, the expression of an *EgCCR1* antisense construct led to a decrease in lignin content. The authors also demonstrated that composite plants were suitable for the analysis of the expression pattern conferred by promoters from genes involved in the lignin biosynthetic pathway.

12.4 Genome Editing in Transgenic Roots of Composite Plants

The first reports of CRISPR/Cas9 editing in plants appeared in 2013, with successful application for both transient expression and recovery of stable transgenic lines. In addition to demonstration of efficacy in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* (Li et al. 2014), there have also been many reports on different crop species including rice (Miao et al. 2013), maize (Liang et al. 2014), and wheat (Wang et al. 2014). Because of the ease of use and low cost, CRISPR/Cas9 has rapidly become the tool of choice for gene editing and creating knockout mutants in plants (Belhaj et al. 2015; Liu et al. 2016; Nogué et al. 2016). However, a prerequisite for the application of the technology is the ability to deliver guide RNAs (gRNAs) and the CRISPR-associated protein 9 to the target cells either stably or transiently. When it is not possible to regenerate edited plants after transient expression of the gRNA and Cas9 in protoplasts and a genetic transformation procedure with *A. tumefaciens* is not available either, composite plants offer an alternative for creating mutations in root-expressed genes (Table 12.3).

The potential of the CRISPR/Cas9 system to induce gene mutations using hairy root transformation was first tested in tomato and targeted the *SHORT-ROOT* (*SHR*)

Table 12.3 Use of genome editing for gene function analysis in composite plants

Plant	Gene	Function	References
Brassica carinata	*FLA1*	Adhesion molecule	Kirchner et al. (2017)
Glycine max	*FEI1, FEI2* *SHR* *GS1* *CHI* *PDS* *Rfg1*	Plant signaling Seed coat development Nitrogen metabolism Flavonoid pathway Carotenoid biosynthesis pathway Resistance to nodulation	Cai et al. (2015) Jacobs et al. (2015) Du et al. (2016) Fan et al. (2017)
Lotus japonicus	*SYMRK*	Symbiotic nitrogen fixation	Wang et al. (2016)
Medicago truncatula	*gus*	Proof of concept	Michno et al. (2015)
Salvia miltiorrhiza	*CPS1* *RAS*	Diterpenoid biosynthesis Phenolic acid biosynthetic pathway	Li et al. (2017) Zhou et al. (2018)
Solanum lycopersicum	*SHORT-ROOT, SCARECROW*	Root development	Ron et al. (2014)
Taraxacum koksaghyz	*1-FFT*	Inulin biosynthesis	Iaffaldano et al. (2016)

1-FFT, fructan 1-fructosyltransferase; CHI, chalcone-flavone isomerase; CPS1, diterpene synthase; FEI1 and FEI2, leucine-rich receptor kinase; FLA1, fasciclin-like arabinogalactan protein 1; GS, glutamine synthase; gus, ß-glucuronidase; PDS, phytoene desaturase; RAS, rosmarinic acid synthase; Rfg1, plant resistance protein; SCARECROW and SHORT-ROOT, Gras transcription factors regulating root patterning; SHR, seed coat wrinkling; SYMK, symbiosis receptor kinase

sequence expressed in root vascular tissue and encoding a transcription factor (Ron et al. 2014). Several hairy roots genetically transformed with a gRNA targeting the coding sequence of *SHR* were obtained and characterized by a short meristem. Sequence analysis of the targeted gene in putatively edited roots confirmed that the *SHR* coding region contained a variety of insertion and deletion (indel) mutations. The alterations in the root phenotype were the result of defects in stem cell division and cell patterning and were consistent with the phenotype of *Arabidopsis shr* mutants. From these data, it was concluded that SHR function was conserved between tomato and *Arabidopsis*.

Similar experiments were performed on the nitrogen-fixing legumes *M. truncatula* (Michno et al. 2015), *G. max* (Cai et al. 2015; Du et al. 2016; Jacobs et al. 2015; Michno et al. 2015; Sun et al. 2015), and *L. japonicus* (Wang et al. 2016). Previously reported ex vitro or in vitro composite plant transformation assays were used to introduce T-DNA gene constructs with the designed gRNA and codon-optimized gene encoding the Cas9 protein. Sequencing of the targeted genes revealed mutations induced by the CRISPR/Cas9 system. Following the analysis of 11 targeted loci in soybean, DNA mutations mainly consisting of small deletions were detected in 95% of the hairy roots (Jacobs et al. 2015). One limitation of the CRISPR/Cas9 system is possible off-target mutations that may alter the expression of genes that were not originally targeted. Experiments on soybean composite plants indicate that off-target mutations do occur, although at low rates (Jacobs et al. 2015).

The CRISPR/Cas9 system is also effective for the study of biosynthetic pathways. Two genes isolated in the Chinese medicinal plant *Salvia miltiorrhiza* coding for water-soluble phenolic acids have been successfully targeted (Zhou et al. 2018). When the diterpene synthase gene *SmCPS1* from the tanshinone biosynthetic pathway was edited, a mutation rate of 42.3% was obtained in the hairy roots, and tanshinone was absent in the homozygous plants (Li et al. 2017). The second gene targeted was rosmarinic acid synthase (*SmRAS*). The level of *RAS* expression was reduced in successfully edited plant roots, revealing a promising potential method to regulate plant metabolic networks and improve the quality of medicinal herbs.

From these recent studies, it can be concluded that CRISPR/Cas9 and related genome editing methods will facilitate a wide range of functional analyses in roots of composite plants, since specific mutations and knockout mutants can be easily obtained, even in non-model plants (Wang et al. 2017).

12.5 Conclusions

The first composite plant obtained after T-DNA transfer with *A. rhizogenes* was reported more than 20 years ago in the legume *L. corniculatus*, the aim being to study genes involved in nodulation with rhizobia. The feasibility and potential of this simple low-cost approach have now been demonstrated in numerous legume and nonlegume plant species. Experiments show that *A. rhizogenes* is a useful tool to rapidly test gene expression and function in the context of root development and in

response to the biotic and abiotic environment. Furthermore, recent findings demonstrate that the CRISPR/Cas9 technology can also be used to induce targeted indel mutations in the root system of composite plants.

While studies on hairy roots advance the speed of the investigations in plants which are also amenable to genetic transformation by *A. tumefaciens*, sometimes they are the only way to obtain gene transfer in plant species that remain recalcitrant to in vitro regeneration and/or T-DNA transfer by *A. tumefaciens* or direct gene techniques. In the future, this system will thus certainly continue to be a valuable way to advance functional genomic research and to improve our knowledge of the molecular mechanisms underlying a wide range of processes in root-microbe and root-parasitic interactions, root development, and root adaptation to abiotic stress.

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