pHairyRed: A Novel Binary Vector Containing the *DsRed2* Reporter Gene for Visual Selection of Transgenic Hairy Roots

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ABSTRACT We developed a new plant transformation vector, *pHairyRed*, for enabling high throughput, non-destructive selection of *Agrobacterium rhizogenes*-mediated 'hairy-root' transformation. *pHairyRed* allows easy *in planta* visualization of transgenic tissue with minimal disturbance to the plant. The *DsRed2* reporter gene, encoding a red fluorescent protein, was cloned to yield *pHairyRed* (harbouring a multiple cloning site), which was used with the highly efficient K599 *A. rhizogenes* strain to infect soybean (*Glycine max* L. Merrill) plants. DsRed2 fluorescence was easily detected *in planta* for the duration of a 5-week study with negligible levels of background autofluorescence. This enabled visual selection of transformed roots and subsequent excission of non-transformed roots. *pHairyRed*-transformed roots nodulated normally when inoculated with *Bradyrhizobium japonicum*. Within the nodule, DsRed2 fluorescence was plant-specific, being absent in the bacteroid-dominated nodule infected zone. To test the reliability of *pHairyRed* as a high-fidelity binary vector reporter system, the gene encoding the soybean Nod factor receptor, *GmNFR1*α, was cloned into the vector for use in a complementation study with a non-nodulating *nfr1*α mutant of soybean. Complementation was achieved and, without exception, DsRed2 fluorescence was detected in all hairy roots that successfully formed nodules (100%, $n = 34$). We anticipate broad application of this reporter system for the further analysis of root-related events in soybean and related legumes.

Key words: Fluorescence imaging; genetics; molecular biology; plant–microbe interactions; soybean; plant transformation.

INTRODUCTION

Agrobacterium rhizogenes-mediated hairy root transformation has led to many significant advances in our understanding of plant molecular biology (e.g. Stiller et al., 1997; Limpens et al., 2003; Estrada-Navarrete et al., 2007; Kereszt et al., 2007; Hayashi et al., 2008; Indrasumunar et al., 2010; 2011). It is a powerful tool that facilitates the integration of novel DNA sequences into the infected host plant. Its extensive use as a reverse-genetics technique has dramatically advanced the understanding of plant development. This is particularly true for many agriculturally and commercially important plant species such as soybean, for which it is often difficult, or time-consuming, to generate whole plants that are stably transgenic.

Hairy root transformation is a relatively simple technique involving the infection of a host plant with a compatible *A. rhizogenes* strain (Beach and Gresshoff, 1988; Kereszt et al., 2007 and references within). This results in the formation of a chimeric transgenic plant with hairy root structures, formed as a result of the expression of the *rol* gene cluster located on the *A. rhizogenes* root-inducing Ri plasmid (Savka et al., 1990). By introducing a novel promoter:reporter gene fusion into the plant, hairy root transformation can determine spatial and temporal aspects of that gene's expression (Martirani et al., 1999; Boisson-Dernier et al., 2001). More can be determined about that gene's role in plant growth and development based on phenotypic, molecular, and/or biochemical differences resulting from overexpression (Vincent et al., 1997; Reid et al., 2011), or RNA interference ('knock-down', Brandwagt et al., 2002) studies.

Despite its many advantages, transformation efficiency of newly generated hairy roots remains a significant drawback of the technique. Whether the T-DNA delivery vector system uses a binary vector (e.g. www.cambia.org, 2009) or integrative

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vectors (e.g. Kereszt et al., 2007), not all generated tissues are transgenic. Furthermore, not all transgenic roots, whether arising from one wound site or from different plants, are generated by equivalent transfer events. Indeed, the resulting expression of the transgene is highly dependent on where and how many times it integrated within the host's genome. This can be extremely problematic in overexpression and RNA interference studies in which scoring transgenic-only tissue is necessary. For example, in RNA interference studies, non-transgenic tissues that are not silenced often result in a 'leaky' response, preventing proper characterization. Efforts have been made to improve the transformation efficiency (Hansen et al., 1994; Kifle et al., 1999; Somers et al., 2003; Kereszt et al., 2007) but it is not possible to attain a 100% transformation efficiency. Therefore, a suitable method for the selection of transgenic tissue is required.

The β-glucuronidase (GUS) reporter system (Jefferson, 1989) is a histochemical assay that has been used extensively in plant research (e.g. Nontachaiyapoom et al., 2007; Hayashi et al., 2008). However, the GUS staining assay is destructive and thus not suitable as a reporter for transgenic hairy root selection in situations in which tissue growth is needed subsequent to screening.

The use of fluorescent proteins provides another method of visualization of tagged tissue. The green fluorescent protein (GFP; Chiu et al., 1996) is widely used as a reporter in prokaryotes and eukaryotes (Tombolini et al., 2006; Nontachaiyapoom et al., 2007). Detection of GFP does not require an external substrate and tissue remains alive after analysis (Chiu et al., 1996). However, detection of GFP in some plant species or organs is problematic due to the autofluorescence emission of lignified and flavone-containing tissues that emit at the same spectral maxima as GFP (Davis and Vierstra, 1998). Although it is possible to separate these respective emissions using spectral unmixing imaging techniques (Berg and Beachy, 2008), as with GUS, this requires the histological fixation and mounting of the tissue (Berg and Beachy, 2008). Due to these complications, GFP is also not suitable for rapid detection of transgenic plant tissue *in vivo*, especially in flavone-rich legume roots.

The red fluorescent protein, DsRed2 (Clontech, www.clontech.com), is an improved variant of DsRed (Clontech, www.clontech.com), derived from the *Discosoma* sp. red fluorescent protein, drFP583 (Matz et al., 1999). Both DsRed and DsRed2 have been used successfully as reporter for plant gene expression and protein localization studies (Limpens et al., 2004, 2005; Tzfira et al., 2005; Nishizawa et al., 2006). DsRed2 is ideal for use in plants because of its high solubility in plant tissues and because its detection does not require a substrate (Nishizawa et al., 2006). The emission spectrum of DsRed2 is significantly different from that of the autofluorescence of plant root tissue, which therefore minimizes background interference (Dietrich and Maiss, 2002; Berg and Beachy, 2008). Another major advantage of DsRed2 is that it is highly resistant to photo-bleaching (Garcia-Parajo et al., 2001), where it maintains high fluorescence levels even following prolonged light exposure (Garcia-Parajo et al., 2001). Furthermore, the *DsRed2* gene is relatively easy to manipulate in vector construction due to its small size of 671 bp.

Here, we describe a new binary transformation vector, *pHairyRed*, containing *DsRed2*, and demonstrate that *pHairyRed* can be used to determine successful transformation events in soybean. Moreover, the *pHairyRed* vector system was tested for genetic complementation of nodulation deficiency using *A. rhizogenes* strain K599-mediated hairy roots generated in the highly important model and crop legume soybean (Ferguson and Gresshoff, 2009).

RESULTS

Successful Construction of the *pHairyRed* Reporter System

To improve the selection of successfully transformed hairy roots, we constructed a new vector, *pHairyRed* (Figure 1A). The *DsRed2* gene (Clontech, www.clontech.com) was inserted in place of the *GUSplus* reporter gene in the binary vector *pCAMBIA 1305.1* (CAMBIA, www.cambia.org). The reporter gene expression cassette is driven by the *CaMV 35S* promoter. For use as an *in planta* reporter system, a castor bean catalase gene intron is situated immediately downstream of the promoter and upstream of *DsRed2* (Figure 1A) and a nopaline synthase poly(A) signal is located at the 3' end of the reporter cassette. These features are highly important to ensure plant-specific reporter gene expression (Tanaka et al., 1990). *pHairyRed* was also designed to contain a multiple cloning site (*pUC18* polylinker) for convenient insertion of further genes of interest. To test the effectiveness of *pHairyRed* as a reporter of transformation events, a *CaMV 35S* promoter-driven *GmNFR1α* overexpression construct was inserted into the *pHairyRed* MCS for use in complementation studies of *nod49*, the soybean non-nodulation *nfr1α* mutant (Mathews et al., 1989a; Indrasumunar et al., 2011).

Agrobacterium rhizogenes K599 Transformed with *pHairyRed*

Agrobacterium rhizogenes strain *K599* (Savka et al., 1990) was transformed with *pHairyRed* and *pHairyRed*::35S-*GmNFR1α* by electroporation. The strain was originally isolated by the late Prof. Alan Kerr of Adelaide (hence the 'K' identifier). Colony PCR analysis using *DsRed2* and *35S-GmNFR1α* primers (see Methods) confirmed transformation success (Figure 1B).

To ensure the *DsRed2* reporter gene system was active in plants, but not in *K599*, epifluorescence analysis was performed (Figure 2C and 2D). An empty *K599* strain (Figure 2A and 2B) that is non-fluorescent was included as a control. The absence of fluorescence in the *pHairyRed K599 strain* (Figure 2D) confirmed that *DsRed2* expression and resulting fluorescence detected from transformed roots is from reporter gene expression *in planta* and is not from *K599* or *K599* carrying *pHairyRed*.

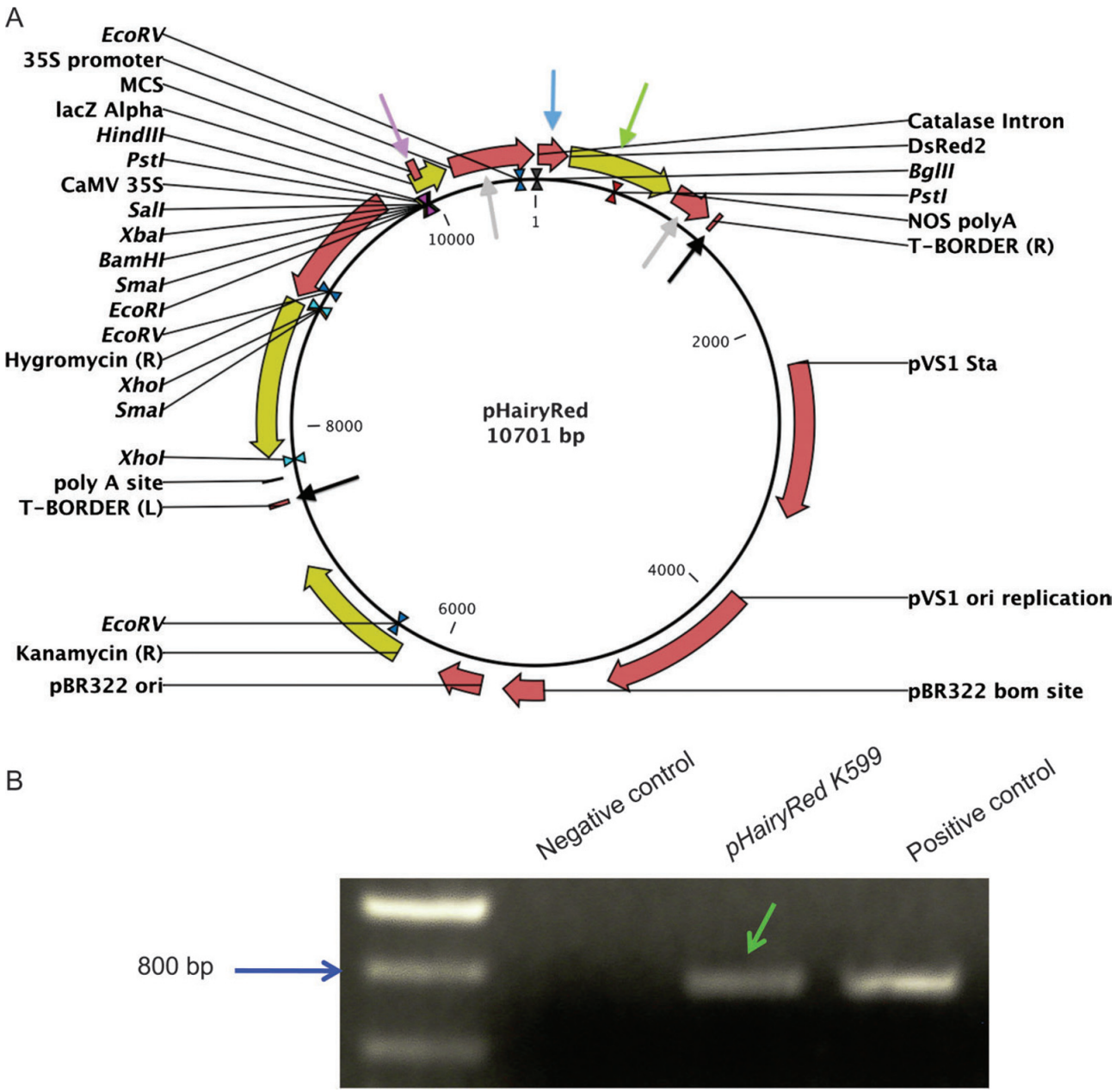

Figure 1. (A) The binary vector, *pHairyRed.* The expression cassette is flanked by the T-DNA left and right borders (black arrows), with *a CaMV 35S* promoter (gray arrow) driving the expression of the *DsRed2* reporter gene (green arrow). The *DsRed2* gene has an upstream *Catalase Intron* castor bean catalase intron (blue arrow) and a downstream *NOS polyA* nopaline synthase terminator (gray arrow). An intact *MCS pUC18* polylinker multiple cloning site (purple arrow) located upstream of the reporter gene construct enables the insertion of additional gene(s).

(B) Colony PCR analysis of the *pHairyRed A. rhizogenes K599 strain.* The amplified fragment (~700 bp) corresponding to *DsRed2* is present in *pHairyRed K599* and the positive control, but is absent in the negative control, confirming the successful transformation of *K599* with *pHairyRed*.

pHairyRed Improves the Detection of Transformation Efficiency

To assess the transformation efficiency of the *pHairyRed* vector, *A. rhizogenes pHairyRed K599* and an empty control *K599* were used in hairy root transformation of soybean cv. Bragg. Emerging roots were screened for DsRed2 fluorescence. Red-fluorescent roots (putative *DsRed2* transgenic) were retained while all non-fluorescent roots were removed by excission. The transformation efficiency (proportion of red-fluorescent roots compared to total) of *pHairyRed* observed 18 d after transformation was 19% (Figure 3). After visual screening and selective root removal, stems were cut below the hairy roots, planted

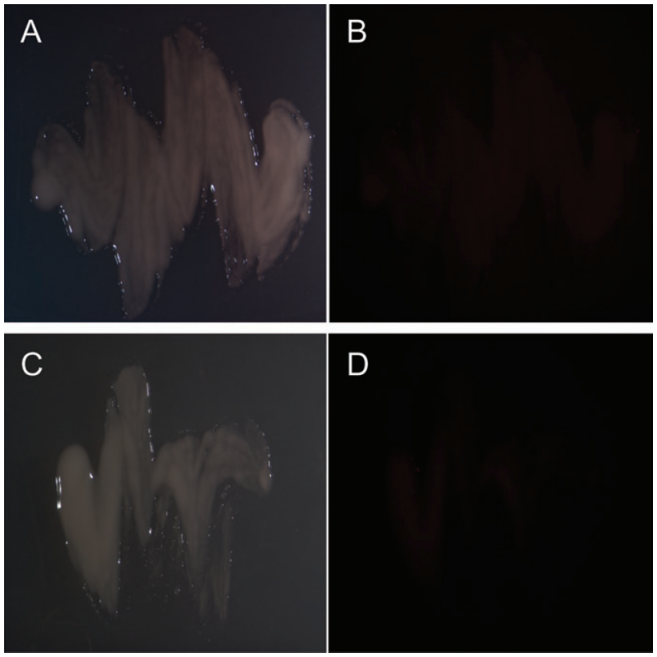

Figure 2. (A, B) Empty *K599* and **(C, D)** *pHairyRed K599* streaks grown on LB agar. (A, C) Bright field analysis reveals no visual difference between the two strains and epifluorescence analysis (B, D) reveals that neither strain exhibits fluorescence, indicating that the reporter expression cassette is inactive in both.

into vermiculite, transferred to glasshouse-grow conditions (see Methods), then inoculated with *B. japonicum* CB1809 after 3 d. At 28 DAI (60 d after transformation), the plants were harvested and their roots were again scored for their fluorescence (i.e. their rate of transgenic expression; Figure 3). In the period following root excision, more roots, both transgenic and non-transgenic, had formed. However, the frequency of transgenic hairy roots was now 34% (Figure 3). This demonstrates that selection using the *pHairyRed* reporter system was highly effective, and although incomplete in increasing the proportion of transgenic tissue present, DsRed2 fluorescence remained stable in all transgenic roots throughout the experiment.

pHairyRed Does Not Interfere with Legume Nodulation

To assess whether DsRed2 in transgenic hairy roots affected physiological function, all hairy roots generated in wild-type, Bragg, plants were inoculated with *B. japonicum CB1809* to induce nodulation. Nodulation of red fluorescent *DsRed2* transgenic roots was compared to that of non-fluorescent, non-transgenic roots. The nodule number per root dry weight of transgenic roots was not significantly different ($p < 0.05$) from that of non-transgenic roots (Figure 4), meaning biological function of *DsRed2* transgenic hairy roots remained unaffected. Furthermore, *DsRed2* expression was detected in both root and nodule tissue (Figure 5) and was confirmed to be plant-tissue-specific due to the absence of DsRed2 fluorescence in the nodule infected zone (Figure 5), which is

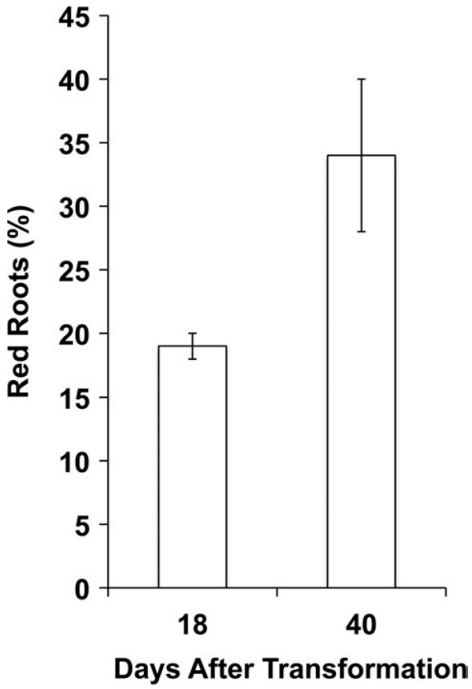

Figure 3. The Percentage of Red Roots Observed Following Soybean Hairy Root Transformation with *pHairyRed*.

By 18 d after transformation, 19% of hairy roots were positive for DsRed2 fluorescence. Following excision of non-fluorescent roots and subsequent re-planting, this increased to 34% by 60 d after transformation.

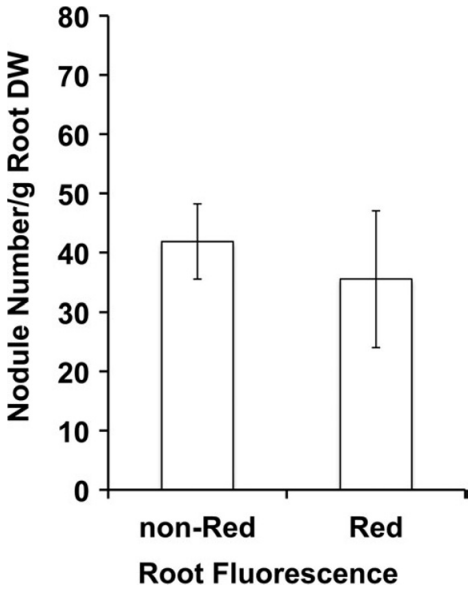

Figure 4. Number of Nodules Formed per Dry Weight of Soybean Hairy Roots Transformed with *pHairyRed*.

No significant difference was detected between non-red (presumed not transformed with *pHairyRed*) and red (presumed transformed with *pHairyRed*) roots ($p < 0.05$).

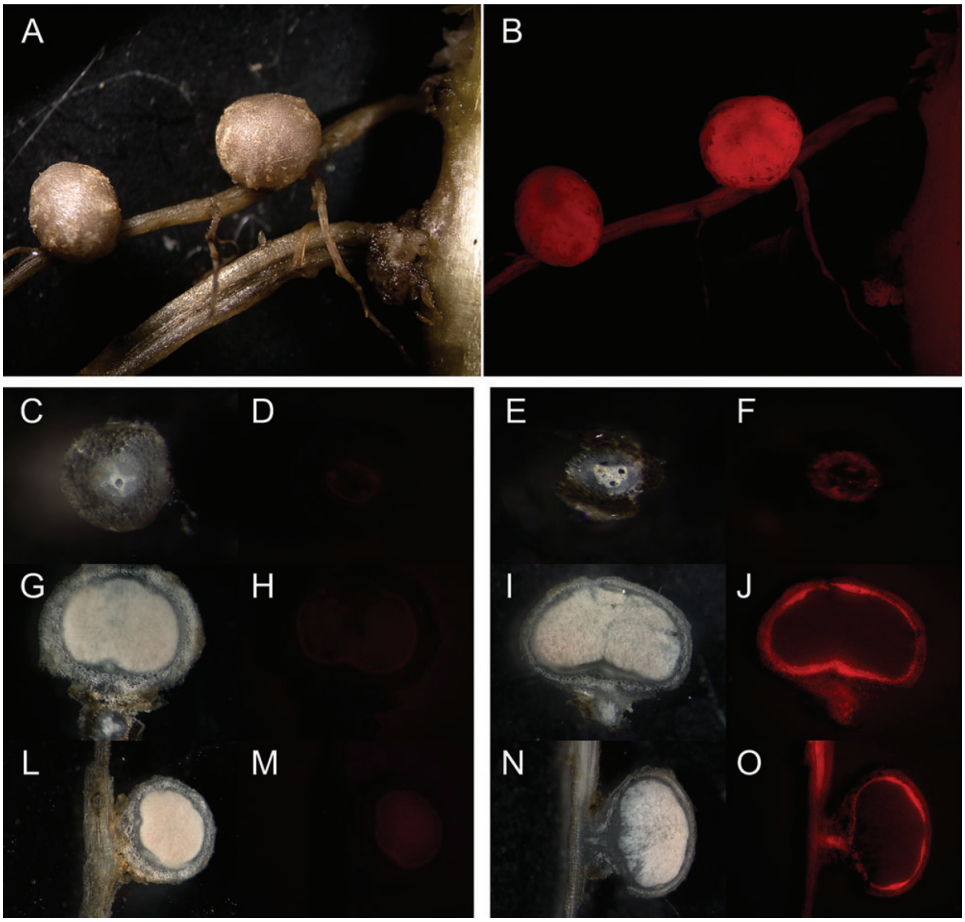

Figure 5. Bright Field and Epifluorescence Images of Soybean Hairy Roots Generated Using Empty *K599* and *pHairyRed*.

(A) Under normal bright field conditions both root types appear similar. However, **(B)** roots and nodules transformed with *pHairyRed* are clearly red when viewed under epifluorescence illumination. **(F, J, O)** Red fluorescence was absent in the xylem of root sections of transgenic hairy roots and **(D, H, M)** in non-transgenic hairy roots. (J, O) In nodules, red fluorescence was not detected in the cortex, which is dominated by bacteroids that are not carrying the *DsRed2* gene and thus not expected to fluoresce. No background or autofluorescence was detected in untransformed plant tissue.

dominated by bacteroid-filled cells (Ferguson et al., 2010). This clear distinction in fluorescence between the two tissue types shows that selection of plant tissue using the *DsRed2* reporter in *pHairyRed* is accurate and consistent. It also shows that the DsRed2 fluorescence emission spectrum does not overlap with that of the autofluorescence of root tissue, meaning background noise is not a problem (Figure 5). Untransformed roots shown in Figure 5D, 5H, and 5M are visible because the camera exposure setting was significantly prolonged to enable the capture of a visible image; it is not root autofluorescence.

Complementation of *nod49* Plants Using *pHairyRed::35S-GmNFR1*α

Legumes, together with soil-dwelling bacteria collectively known as rhizobia, can form a symbiosis that leads to the formation of specialized root structures called nodules, in which the rhizobia fix atmospheric nitrogen gas for the plant (Udvardi et al., 1988; Ferguson et al., 2010). A critical step in nodule formation is a signal exchange between the plant and bacteria (Ferguson and Mathesius, 2003) that includes the production of a rhizobia-secreted nodulation signal, Nod Factor (NF; Dénarié et al., 1996). NF is subsequently perceived by plant LysM receptor kinases called Nod factor receptors (NFR) (Radutoiu et al., 2003; Indrasumunar et al., 2010, 2011). In soybean, these receptors have been designated *NRF1*α/β and *NFR5*α/β.

The specificity of *DsRed2* as a transgenic hairy root marker was assessed by transforming the soybean non-nodulating *nfr1*α mutant *nod49* (Mathews et al., 1989a, 1989b, 1992) with the *GmNFR1*α overexpression *A. rhizogenes* strain *pHairyRed*::*35S-GmNFR1*α *K599*. Hairy roots detected with DsRed2 fluorescence were expected to nodulate as a result of *GmNFR1*α overexpression while non-transgenic hairy roots were expected to have the mutant non-nodulating phenotype.

Hairy roots from individual mutant plants (n = 15) were evaluated for both DsRed2 fluorescence and nodule number (Figure 6C). All hairy roots (n = 34) detected with DsRed2 fluorescence were successfully complemented with *GmNFR1*a and

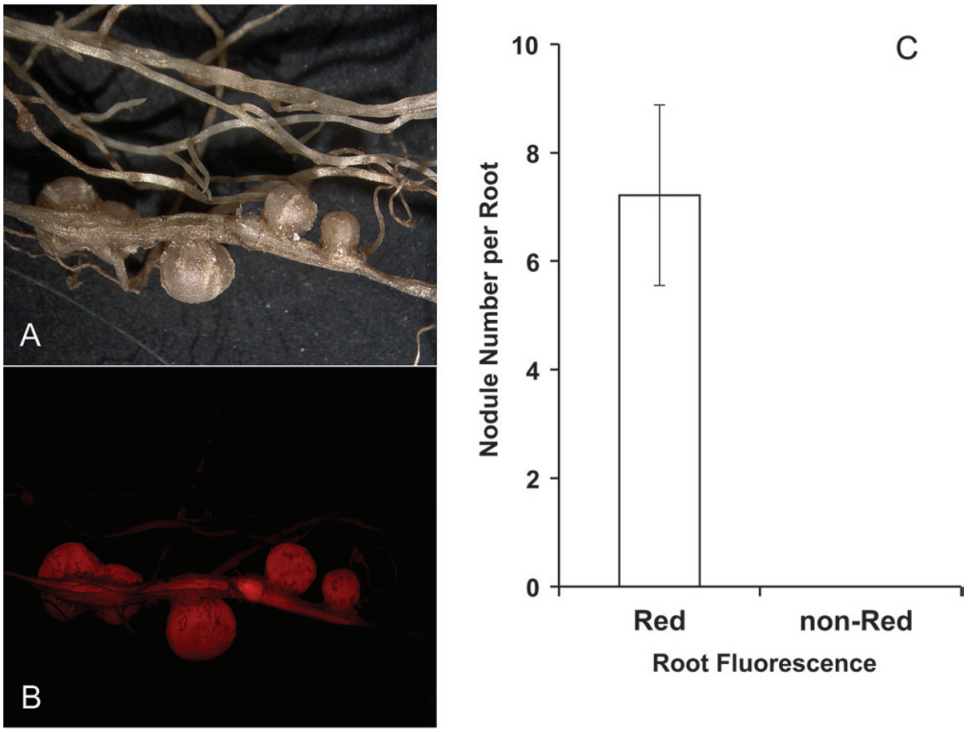

Figure 6. (A) Bright field and **(B)** epifluorescence images of hairy roots formed on the non-nodulating *nfr1α* mutant of soybean. The hairy roots were generated using *K599* harboring the *GmNFR1α* overexpression vector *pHairyRed*::*35S-GmNFR1α*. (A) Transgenic and non-transgenic roots appear the same under normal light. However, under epifluorescence illumination, (B) roots presumed to be transgenic are red while those presumed to be non-transgenic roots are not. (B) Non-fluorescent roots did not exhibit any nodule structures, whereas all fluorescent roots did, indicating successful *GmNFR1α* complementation.

formed nodules while all non-fluorescent roots displayed the mutant phenotype and failed to form nodules (Figures 6A–6C, and 5A and 5B). Similar results have been observed in subsequent experiments using *pHairyRed* (Lin, 2010, data not shown). This demonstrates clearly that *pHairyRed* can be used to deliver transgenes into plants, that all fluorescent roots are indeed transgenic, and that DsRed2 fluorescence is not detected in non-transformed roots.

DISCUSSION

Here, we introduce the novel binary vector, *pHairyRed*, which incorporates the *DsRed2* reporter to assess visually the efficiency of hairy root transformation events in soybean. *pHairyRed* allows non-destructive monitoring of living tissue at any given time and with minimal disturbance to the plant. Moreover, it can potentially be used to transform a wide range of scientifically and agronomically important plant species, including *Glycine max*, *Phaseolus vulgaris*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *Lotus japonicus*, and *Medicago truncatula*.

Reverse-genetic approaches in plants, such as RNA interference and gene overexpression, have employed conventional reporters such as GUS (Hayashi et al., 2008) and the fluorescent proteins GFP (Seki et al., 2005) and YFP (Tomilov et al., 2007). However, these reporters are not suitable for use in fast, reliable detection of transformation events in living tissues. In contrast, DsRed2 is an ideal reporter for such studies. It is a small protein, is soluble in plant tissue (Nishizawa et al., 2006), and its fluorescence is highly resistant to photo-bleaching (Garcia-Parajo et al., 2001). It is a relatively fast maturing chromophore (<24 h; Bevis and Glick, 2002) and fluorescence is detected consistently from very early to very late time points following transformation. DsRed2 detection is highly accurate, substrate-less, non-invasive, and does not require histological preparation. This, coupled with simple *in planta* detection, allows repeated monitoring of treated plant tissue. DsRed2 does not share the inherent limitations of other conventional reporters, meaning *pHairyRed* can facilitate a number of experimental approaches, such as those required for many reverse-genetic studies, translational-fusions for protein localization studies, and various studies involving promoter substitutions. DsRed has also been used in hairy root transformation studies (Limpens et al., 2004). However, its lower solubility and slower maturation time compared with DsRed2 may make detection of very early developmental events more difficult.

We demonstrate the application of *pHairyRed* for simple, rapid, and repeatable selection of living, transformed soybean hairy roots. The hairy roots remain unaffected by the screening process and grow normally after replanting. The resultant chimeric ('composite') plants successfully formed nodules after inoculation with *B. japonicum CB1809*. Furthermore, *pHairy*

Red::35S-*GmNFR1α* transformation of the soybean non-nodulating *nfr1α* soybean mutant, *nod49*, demonstrated the successful complementation of the mutant phenotype by completely recovering nodulation function. Moreover, *pHairyRed* selection of complemented hairy roots was 100% reliable, as all fluorescent hairy roots formed nodules while all non-fluorescent hairy roots did not.

METHODS

Bacterial Growth Conditions

E. coli strain *DH5α* was transformed by electroporation with various constructs and grown at 37°C on LB agar (tryptone 10 g L^{-1}; yeast extract 5 g L^{-1}; NaCl 5 g L^{-1}; pH = 7.5; 1.5% agar) plates containing relevant antibiotics for selection.

Agrobacterium rhizogenes strain *K599* (Savka et al., 1990) was grown for 48 h at 28°C on minimal medium (K_2HPO_4 0.5 g L^{-1}; $MgSO_4$•$7H_2O$ 0.2 g L^{-1}; NaCl 0.1 g L^{-1}; mannitol 10 g L^{-1}; $(NH_4)_2SO_4$ 1 g L^{-1}; malic acid 1.34 g L^{-1}; biotin 1 mg ml^{-1}; Broughton and Dilworth trace elements (Broughton and Dilworth, 1971); pH = 7.2; 1.5% agar) agar plates containing 30 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin.

Bradyrhizobium japonicum strain *CB1809* was grown in YMB (mannitol 2 g L^{-1}; yeast extract 0.4 g L^{-1}; K_2HPO_4 0.5 g L^{-1}; $MgSO_4$•$7H_2O$ 0.2 g L^{-1}; NaCl 0.1 g L^{-1}; pH = 7) medium for 48 h at 28°C. Cultures were diluted with water to a final concentration of OD_{600} = 0.1 prior to inoculating plants.

Plant Growth Conditions

Soybean *Glycine max* (L. Merr.) wild-type cv. Bragg and the non-nodulating *nfr1α* mutant, *nod49*, were used. *nod49* has a point mutation in exon 5 of the *GmNFR1α* gene (Indrasumunar et al., 2011) which results in loss of function and a non-nodulation phenotype. Seeds were surface-sterilized by immersing in 70% ethanol for 30 s, then rinsed five times with sterile water. Seeds were sown in sterilized grade 2 vermiculite in 4-L pots. Plants were watered with the Reid-modified Herridge (RMH) nutrient solution (Herridge, 1977) (KH_2PO_4 1 mM; K_2HPO_4 1 mM; $MgSO_4$•$7H_2O$ 2 mM; KCl 1.5 mM; $CaCl_2$•$2H_2O$ 2.5 mM; Fe(III)-EDTA 34.8 mg L^{-1}; H_3BO_3 2.86 mg L^{-1}; $MnCl_2$•$4H_2O$ 1.812 mg L^{-1}; $ZnCl_2$ 0.112 mg L^{-1}; $CuCl_2$•$2H_2O$ 0.052 mg L^{-1}; Na_2MoO_4•$2H_2O$ 0.024 mg L^{-1}) every 2 d. Conviron model E7/2 growth chambers were maintained at 27°C/23°C 16 h/8 h day/night conditions with 80% relative humidity. Glasshouses growth conditions were controlled at a 28°C/23°C 16 h/8 h day/night cycle.

pHairyRed Plasmid Construction

The *DsRed2* gene was PCR amplified from the plasmid *pDsRed2* as a *Spe*I–*Pml*I fragment using the primers (restriction site underlined): DsRed2SpeI_F 5′- CATGCAACTAGTATGGCCTC-CTCCGAGAAC-3′, DsRed2Pml_R 5′- TCCAAGCACGTGGCTACA-GGAACAGGTG-3′. The *DsRed2* PCR fragment was then subcloned into the TA cloning vector, *pCR2.1–TOPO* (InVitrogen, www.invitrogen.com/). The resulting plasmid was double-digested with *Spe*I–*Pml*I, resulting in a *DsRed2* fragment with a 5′–*Spe*I sticky end and 3′–*Pml*I blunt end.

The binary vector, *pCAMBIA 1305.1* (CAMBIA, www.cambia.org), was double-digested with *Spe*I–*Pml*I, effectively removing the *GUSplus* reporter. The *Spe*I–*Pml*I-digested *DsRed2* fragment was then ligated into the corresponding *Spe*I–*Pml*I-digested *pCAMBIA 1305.1* plasmid. The resultant *DsRed2*-containing *pCAMBIA 1305.1* plasmid is designated *pHairyRed*.

pHairyRed::GmNFR1α Plasmid Construction

A *pHairyRed*-based *GmNFR1α* overexpression binary vector was constructed for *nod49* complementation. The overexpression construct consisted of the *GmNFR1α* gene with the *CaMV 35S* promoter placed immediately upstream to drive its expression. The *35S–GmNFR1α* construct was amplified as a *Sac*I–*Sal*I fragment from the plasmid *F522* (Indrasumunar et al., 2011) using the primers (restriction site underlined): 35S_SacI_F 5′-GCTAGAGAGCTCAACATGGTGGAGCA-3′, GmNFR1a_SalI_R 5′-CGATAGGTCGACTCATCTCACAG-3′. The resulting fragment was subcloned into the TA cloning vector, *pCR2.1–TOPO* (InVitrogen, www.invitrogen.com/). This plasmid was *Sac*I–*Sal*I double digested, resulting in a *35S–GmNFR1α* fragment with 5′–*Sac*I and 3′–*Sal*I sticky ends. This fragment was then ligated into a *Sac*I–*Sal*I-digested *pHairyRed* plasmid to form the *GmNFR1α* overexpression vector, *pHairyRed*::*GmNFR1α*.

Agrobacterium rhizogenes Strain Generation

Agrobacterium rhizogenes strain *K599* (Savka et al., 1990) was transformed with respective binary vectors by electroporation and grown at 28°C on minimal medium (K_2HPO_4 0.5 g L^{-1}; $MgSO_4$•$7H_2O$ 0.2 g L^{-1}; NaCl 0.1 g L^{-1}; mannitol 10 g L^{-1}; $(NH_4)_2SO_4$ 1 g L^{-1}; malic acid 1.34 g L^{-1}; biotin 1 mg ml^{-1}; Broughton and Dilworth trace elements (Broughton and Dilworth, 1971); pH = 7.2; 1.5% Difco-Bacto agar) agar plates containing 30 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin.

Hairy Root Transformation and Complementation Studies

The *A. rhizogenes K599* strains harboring *pHairyRed* and the *nod49* complementation plasmid *pHairyRed*::35S–*GmNFR1α*, respectively, were used to infect soybean cv. Bragg in hairy root transformations using the hypocotyl stabbing method (Kereszt et al., 2007). After successful hairy root formation (18 d after transformation), stems were cut just below the infection site and individual plants were screened (described in detail below) for transgenic (fluorescent red) roots. Non-transgenic roots were removed using a sterile scalpel. After screening, plants having only transgenic roots were replanted and maintained for a further 7 d under the same growth chamber conditions as described in Kereszt et al. (2007), but no longer covered with humidity control domes.

After 7 d, plants were transferred to vermiculite in individual 4-L pots and watered immediately with (RMH) nutrient solution. Plants were then transferred to a climate-controlled

glasshouse from this point (16:8 h light:dark and 26:22°C regime).

Three days after replanting, the plants were inoculated with a 1:1 water-diluted *Bradyrhizobium japonicum* CB1809. Plants remained in glasshouse conditions for 4 weeks after inoculation and were watered every 2 d with RMH nutrient solution.

Epi-Fluorescence Visualization of DsRed2

DsRed2 fluorescence was detected using a Nikon model SMZ800 stereoscopic zoom microscope (Nikon) equipped with an epi-fluorescence attachment mounted with a 41002c TRITC (Rhodamine with narrow-band excitation filter) Red Shifted Emission filter (Chroma Corp.) for 530–560 nm excitation and 590–650 nm emission.

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