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*.