



The non-homologous end-joining pathway is involved in stable transformation in rice

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Stable transformation with T-DNA needs the coordinated activities of many proteins derived from both host plant cells and *Agrobacterium*. In dicot plants, including *Arabidopsis*, it has been suggested that non-homologous end-joining (NHEJ)—one of the main DNA double-strand break repair pathways—is involved in the T-DNA integration step that is crucial to stable transformation. However, how this pathway is involved remains unclear as results with NHEJ mutants in *Arabidopsis* have given inconsistent results. Recently, a system for visualization of stable expression of genes located on T-DNA has been established in rice callus. Stable expression was shown to be reduced significantly in NHEJ knock-down rice calli, suggesting strongly that NHEJ is involved in *Agrobacterium*-mediated stable transformation in rice. Since rice transformation is now efficient and reproducible, rice is a good model plant in which to elucidate the molecular mechanisms of T-DNA integration.

Keywords: *Agrobacterium*, non-homologous end-joining, rice, stable transformation, T-DNA

INTRODUCTION

Agrobacterium tumefaciens enables genetic transformation of many plant species via the movement of transferred-DNA (T-DNA) of Ti plasmids into the plant nucleus. Despite its use in both basic research and molecular breeding in several crops, many species and varieties are still recalcitrant to *Agrobacterium*-mediated transformation. To overcome this limitation requires not only optimization of cell and tissue culture conditions but also elucidation of the molecular mechanisms of all the events that occur during *Agrobacterium*-mediated transformation.

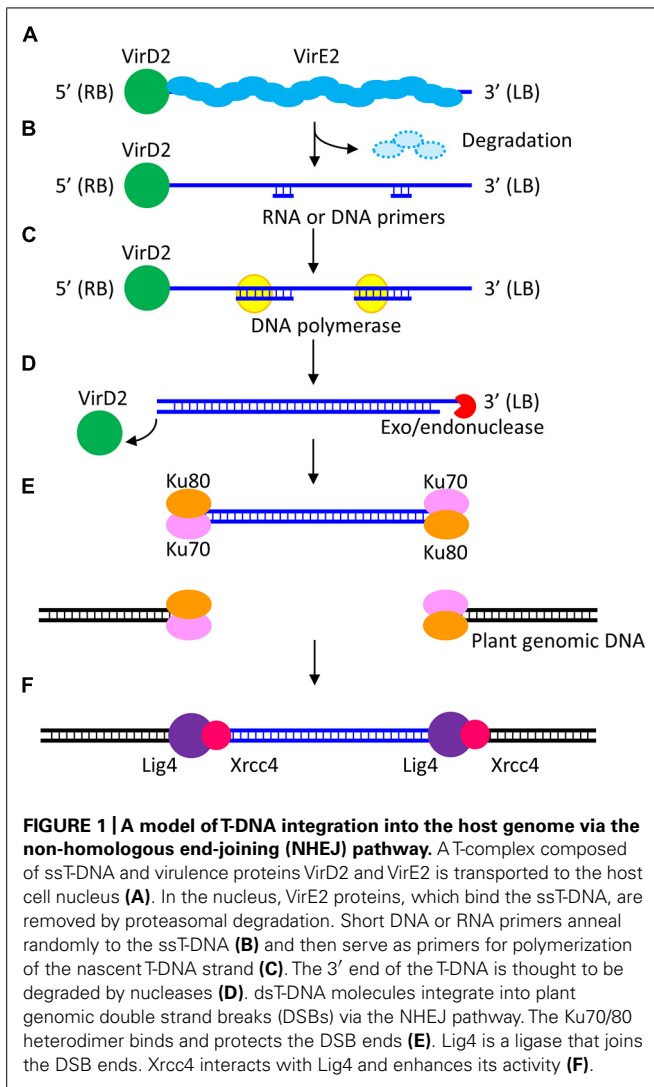
In the conventional *Agrobacterium*-mediated transformation system in rice, callus derived from the scutellum of mature seeds or immature embryos is generally used for *Agrobacterium* inoculation (Nishimura et al., 2006; Hiei and Komari, 2008). Since these tissues have a stereo architecture consisting of many cells, *Agrobacterium* can only infect cells on the surface, and, unlike protoplasts, it is quite difficult to isolate single cells from these tissues without any selection pressure. Indeed, there are far fewer transformed than non-transformed cells when *Agrobacterium*-inoculated primary calli derived from mature seeds are cultured without selection pressure in a conventional transformation system (Saika and Toki, 2009; Saika et al., 2011b). Therefore, transformed cells in which antibiotic- and herbicide-resistance genes located on T-DNA are present, but not expressed to a sufficient level, cannot be distinguished from non-transformed cells.

Agrobacterium-mediated transformation has many steps (Gelvin, 2010; Pitzschke and Hirt, 2010; Magori and Citovsky, 2011; Shibleth and Tzfira, 2012). The last step, i.e., stable transgene expression that results from T-DNA integration into the host genome (and the avoidance of the gene silencing system) is crucial to the clonal propagation of transformed cells of rice as referred to above. Meanwhile, transient expression of transgenes is often observed at an earlier stage when transgenes

are expressed stably and constantly from T-DNA that has not integrated into the rice genome. After import into the nucleus of the T-complex, which consists of single stranded T-DNA (ssT-DNA) and proteins such as virD2 and virE2, ssT-DNA is replicated to double-strand T-DNA (dsT-DNA). Hypothetical models of dsT-DNA formation have been proposed as shown in **Figure 1** (Liang and Tzfira, 2013). Subsequently, transgenes located on the dsT-DNA are expressed. However, this expression is not continuous because naked T-DNA that has not integrated into the host genome is susceptible to degradation (Gelvin, 2010). The transient expression of a cytokinin biosynthesis gene, which leaves no selection marker or vector backbone in the host genome, has been exploited in a transformation system for *Solanaceous* plants (Richard et al., 2008). However, it is thought that transient transgene expression is not crucial to the production of clonally propagated transformed cells in the case of rice.

MOLECULAR MECHANISM OF STABLE TRANSFORMATION VIA *Agrobacterium* IN *Arabidopsis*

The molecular mechanisms of T-DNA integration into the plant genome in *Agrobacterium*-mediated transformation remain unclear. The sequences of T-DNA integration sites were determined in *Arabidopsis* and tobacco plants over 20 years ago (Gheysen et al., 1991; Mayerhofer et al., 1991). Based on these pioneer reports, two major models—the strand-invasion model and the double strand break (DSB) repair model—were proposed (Gelvin, 2010; Pitzschke and Hirt, 2010; Magori and Citovsky, 2011; Shibleth and Tzfira, 2012). In the strand-invasion model, T-DNA integration is thought to be caused by microhomology-mediated repair between ssT-DNA and the plant genome. On the other hand, in the DSB repair model, dsT-DNA is thought to integrate via non-homologous



end-joining (NHEJ)—one of the main DSB repair pathways—into the DSB sites that occur randomly in the plant genome (Figure 1).

Many proteins involved in the NHEJ pathway, such as Ku70, Ku80, Ligase 4 (Lig4), and Xrcc4, have been characterized in model plants such as *Arabidopsis* and rice (West et al., 2000; Riha et al., 2002; Kimura and Sakaguchi, 2006; Hong et al., 2010; Nishizawa-Yokoi et al., 2012). To summarize previous reports in *Arabidopsis*, on the whole, the frequency of stable transformation tends to decrease in NHEJ mutants except for *xrcc4*, although this point remains controversial. It was reported initially that the frequency of stable transformation increased twofold in *in planta* floral dip transformation experiments in *Arabidopsis ku80* mutants (Gallego et al., 2003), while another report demonstrated a two to threefold decrease (Friesner and Britt, 2003). However, a root tumorigenesis assay in *Arabidopsis ku80* mutants confirmed that the stable transformation frequency was decreased severely, although the frequency of transient transformation remained comparable to controls (Li et al., 2005b). In addition, overexpression of Ku80 enhanced stable but not transient transformation frequency (Li

et al., 2005b). Moreover, recent reports have shown that stable transformation frequency was decreased two to fourfold in *Arabidopsis ku80* mutants in both root tumorigenesis assay and in *in planta* floral dip assay (Jia et al., 2012; Mestiri et al., 2014), and stable transformation frequency was also decreased fourfold in *Arabidopsis ku70* mutants in the *in planta* floral dip assay (Jia et al., 2012). Thus, *Arabidopsis* Ku70/80 plays a positive role in stable transformation. It was proposed that Ku80 proteins interact with dsT-DNA molecules and direct them to DSB sites (Li et al., 2005b). On the other hand, there are contrary reports in *Arabidopsis lig4* mutants: some authors found that stable transformation frequency was decreased to 40–70% in *in planta* floral dip assay (Friesner and Britt, 2003), but others found comparable rates to wild-type in both root tumorigenesis assay and *in planta* floral dip assay (van Attikum et al., 2003). These inconsistencies are probably due to differences in cell type (somatic cells vs germ cells) and/or experimental procedures (Gelvin, 2010; Nishizawa-Yokoi et al., 2012; Lacroix and Citovsky, 2013). Interestingly, the possibility that Xrcc4 plays a negative role in stable transformation via interaction with virE2 has been raised (Vaghchhipawala et al., 2012). In this latter theory, active Xrcc4 proteins are inhibited by virE2 proteins and T-DNA is integrated into DSB sites that are either not repaired or that occur *de novo* by suppression of the NHEJ pathway at a higher frequency. *Agrobacterium* might bring stable transformation to a successful conclusion by the good use of host proteins involved in the NHEJ pathway, among others Ku80 and Xrcc4. Just recently, Mestiri et al. (2014) reported that DSB repair pathways including NHEJ are redundantly involved in stable transformation. Stable transformation frequencies were decreased in the single mutants, *ku80*, *xrcc1*, *xrcc2*, and *xpf* (Mestiri et al., 2014), which are deficient in NHEJ, Ku-independent NHEJ (known as alternative NHEJ to distinguish it from classical Ku-dependent NHEJ), microhomology-mediated end joining/single strand annealing and homologous recombination, respectively. Interestingly, stable transformation was markedly but not completely suppressed in a quadruple mutant (Mestiri et al., 2014), possibly because the quadruple mutant did not lose DSB repair activity completely (Charbonnel et al., 2011).

MOLECULAR MECHANISM OF STABLE TRANSFORMATION VIA *Agrobacterium* IN RICE

Unlike *Arabidopsis*, rice is not a natural host plant of *Agrobacterium*; however, the combination of a sophisticated tissue culture system and activation of *Agrobacterium* using the phenolic compound acetosyringon, allows transgenic rice plants to be produced via this method (Hiei et al., 1994). Human cells and yeast—also not natural hosts of *Agrobacterium*—are also susceptible to *Agrobacterium*-mediated transformation (Bundock et al., 1995; Kunik et al., 2001). Moreover, the NHEJ pathway is involved in T-DNA integration in yeast (van Attikum et al., 2001; van Attikum and Hooykaas, 2003). These findings suggest that *Agrobacterium* uses a fundamental DNA repair system that is common to both host and non-host organisms, including rice, in the step leading to stable transformation. Since no conventional root transformation system or *in planta* floral dip transformation system has been established in rice, we are currently restricted to rice callus for

analyzing stable transformation frequencies. Tumorigenesis assays are not applicable to rice callus because this tissue has already dedifferentiated. In *Arabidopsis* and *Nicotiana*, stable transformation frequencies are estimated by visualization of transformed cells (Mysore et al., 1998; Anand et al., 2007). In rice, transient and stable transgene expression can also be observed using green fluorescent protein—a non-destructive and visible selection marker (Toki et al., 2006; Saika et al., 2012). Visualization of transformed cells can quantify stable transformation events rapidly and easily compared to clonal propagation of transformed cells using antibiotics. We have established a sequential monitoring system for stable transformation events in rice callus that uses the enhancer trap of the click beetle luciferase gene, which is 30 times more sensitive than firefly luciferase (Saika et al., 2012). Using this system, we showed that the stable transformation frequency is decreased in knock-down lines of *OsKu70*, *OsKu80*, and *OsLig4* genes and the knock-out line of the *OsKu70* gene (Nishizawa-Yokoi et al., 2012; Saika et al., 2012), suggesting that the NHEJ pathway is involved in the stable transformation process also in rice.

PROSPECTS

According to the DSB repair model, DSBs could occur in cells successfully transformed with T-DNA. In *Arabidopsis*, DSBs induce cell death and endocycle, which halts cell division (Adachi et al., 2011). Cells in which these events occur cannot propagate clonally if stable transformation is successful. Thus, transformation frequency in *Arabidopsis* might be underestimated in mutants in which DSBs occurs at higher frequency, such as NHEJ deficient mutants. However, in rice callus, endocycle does not occur even under genotoxic conditions inducing DSBs (Endo et al., 2012). Thus, transformation frequency may be estimated more accurately in rice callus than in *Arabidopsis*.

Transformation frequency in *Arabidopsis* and rice depends on the variety (Nam et al., 1997; Saika and Toki, 2010). Furthermore, optimal conditions for cell and tissue culture differ among rice varieties (Hiei and Komari, 2008). For example, Koshihikari—an elite variety from Japan—shows lower regeneration frequency due to lower activity of nitrite reductase (Nishimura et al., 2005; Ozawa and Kawahigashi, 2006). This makes it difficult to compare transformation frequency precisely among rice varieties since different callus culture conditions can affect *Agrobacterium* activity and growth. Thus, in order to compare transformation frequency precisely, experiments must be performed under reliable, reproducible and identical conditions using mutants with the same genetic background. Recent technological advances have made it much easier to produce plants with knockouts of targeted genes: successful gene knockouts using artificial nucleases such as transcription activator-like effector nucleases (TALENs) and CRISPR/Cas have already been reported in rice (Li et al., 2012; Feng et al., 2013; Jiang et al., 2013; Miao et al., 2013; Shan et al., 2013; Xu et al., 2014). A series of mutants in which genes involved in the NHEJ pathway are disrupted can now be produced easily in the same variety. Moreover, targeted mutagenesis using artificial nucleases enables “null” and “truncated” mutants to be produced as necessary, unlike conventional mutagenesis approaches and T-DNA tagging lines. These new technologies could help

resolve some of the conflicting results in this field. For example, the involvement in stable transformation of the *Arabidopsis lig4* mutant described above remains controversial, and conflicting results have also been reported in mutants in pathways other than NHEJ. For example, the plant protein VirE2 binding protein 1 (VIP1) has long been considered important in transient and/or stable transformation (Tzfira et al., 2001, 2002; Li et al., 2005a), but results that dispute this view have been reported recently (Shi et al., 2014). A study using knockout mutants of *Lig4* and *Vip1* in rice produced by artificial nucleases could lead to an answer.

Gene targeting (GT) is a transformation technology that can modify a targeted gene in a predicted manner. Successful examples of gene modification via GT have been reported in several higher plants including rice and *Arabidopsis*. However, rice is currently the only flowering plant in which target modification via GT can be performed routinely (Endo et al., 2007; Saika et al., 2011a; Nishizawa-Yokoi et al., 2014; Osakabe et al., 2014). In particular, GT using positive-negative selection enables the introduction of desirable mutations that cause amino acid substitutions in the targeted rice gene (Wakasa et al., 2012; Dang et al., 2013; Nishizawa-Yokoi et al., 2014). Molecular analyses and structure-based protein engineering can reveal the essential amino acids involved in protein–protein interactions. For example, as mentioned above, *Agrobacterium* virE2 protein interacts with Xrcc4 proteins (Vaghchhipawala et al., 2012). It is easy to produce mutant rice plants in which mutated Xrcc4 proteins are expressed. The comparison of transient/stable transformation frequencies in *xrcc4* mutant rice lines deficient in the interaction with virE2 protein or the enhancement of Lig4 activity will be able to provide clues as to the exact roles of Xrcc4 protein in stable transformation. Similarly, a set of rice mutants expressing mutated proteins that have lost the ability to interact with proteins derived from *Agrobacterium* will be useful in analyzing transformation frequency. Such studies will offer new insights into the stable transformation process. Moreover, further analysis will enable the discovery of chemicals effective in strictly activating or suppressing stable transformation, and these could be applied not only in experimental procedures in plants that are recalcitrant to *Agrobacterium*-mediated transformation but also in cases where T-DNA integration is not desired, such as site-directed mutagenesis using artificial nucleases and GT.

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