

Involvement of KU80 in T-DNA integration in plant cells

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In *Agrobacterium*-mediated genetic transformation of plant cells, the bacterium exports a well defined transferred DNA (T-DNA) fragment and a series of virulence proteins into the host cell. Following its nuclear import, the single-stranded T-DNA is stripped of its escorting proteins, most likely converts to a double-stranded (ds) form, and integrates into the host genome. Little is known about the precise mechanism of T-DNA integration in plants, and no plant proteins specifically associated to T-DNA have been identified. Here we report the direct involvement of KU80, a protein that binds dsT-DNA intermediates. We show that *ku80*-mutant *Arabidopsis* plants are defective in T-DNA integration in somatic cells, whereas KU80-overexpressing plants exhibit increased susceptibility to *Agrobacterium* infection and increased resistance to DNA-damaging agents. The direct interaction between dsT-DNA molecules and KU80 *in planta* was confirmed by immunoprecipitation of KU80 dsT-DNA complexes from *Agrobacterium*-infected plants. Transformation of KU80-overexpressing plants with two separate T-DNA molecules resulted in an increased rate of extrachromosomal T-DNA to T-DNA recombination, indicating that KU80 bridges between dsT-DNAs and double-strand breaks. This last result further supports the notion that integration of T-DNA molecules occurs through ds intermediates and requires active participation of the host's nonhomologous end-joining repair machinery.

Agrobacterium | DNA repair | double-strand breaks

A *Agrobacterium* is the only known organism capable of transkingdom DNA transfer (1) to a wide number of species from different genera. These include plants (reviewed in refs. 2 and 3), yeast (4), fungi (5, 6), and even human cells (7). The genetic transformation process begins with the transfer of a single-stranded (ss) copy (T-strand) of the transferred DNA (T-DNA) segment located on the bacterial tumor-inducing plasmid and several virulence (Vir) proteins into the host cell and ends with integration of the T-DNA into the host cell genome (2, 8). T-DNA travels into the host cell as a protein-DNA complex (T-complex), with a single VirD2 attached to the 5' end of the T-strand. Inside the host cell, the remainder of the T-strand is thought to be packed with numerous molecules of VirE2, a ssDNA-binding protein (reviewed in refs. 8 and 9). The T-complex is proposed to be imported into the host cell nucleus by interaction of the VirE2 and VirD2 proteins with the bacterial protein VirE3 (10) and the host factors VIP1 (11) and importin α (12). Once inside the nucleus, the T-complex is stripped of its escorting proteins, VirE2 and VIP1, by targeted proteolysis (13) and converts into a double-stranded (ds) intermediate, before its ultimate integration into the host genome, most likely as a ds molecule (14, 15).

It is currently accepted that *Agrobacterium* Vir proteins do not possess any known DNA-repair activity, and T-DNA integration is therefore most likely effected by host proteins (16). Under this scenario, the host cell DNA-repair machinery recognizes dsT-DNA molecules as genomic ds breaks (DSBs) and mediates their integration by a DNA-repair mechanism. Indeed, the roles of

various DNA-repair proteins have been studied in yeast-based systems, demonstrating that key DNA-repair proteins are essential for T-DNA integration into the yeast cell genome by either homologous or nonhomologous (illegitimate) recombination (HR and NHR, respectively) (17, 18). Because in plant cells, T-DNA integration occurs mainly through NHR, it is most likely that nonhomologous end-joining (NHEJ) proteins will be required for the process of T-DNA integration. Interestingly AtLIG4, a NHEJ plant protein found to be important for T-DNA integration in yeast cells, is not required for T-DNA integration in plants (19). Moreover, the involvement of AtKU80, a key NHEJ protein, in T-DNA integration is inconclusive, because it has been reported to be both required (20) and dispensable (21) for T-DNA integration. Note that the latter contradictory results may simply have arisen from the nature of the flower-dip transformation method, which is performed under relatively uncontrolled conditions. Thus, the question of which host factors control T-DNA's integration in somatic cells remains open. Here we show that *ku80*-mutant *Arabidopsis* plants are defective in T-DNA integration in somatic cells, whereas KU80-overexpressing plants exhibit increased susceptibility to *Agrobacterium* infection and increased resistance to DNA-damaging agents. We also demonstrate a direct link between dsT-DNA molecules and KU80 *in planta* and show that overexpression of KU80 in transgenic plants increases the rate of extrachromosomal T-DNA to T-DNA recombination, thus indicating a function for KU80 in bridging between dsT-DNAs and DSBs.

Materials and Methods

Plant Materials, Tumorigenesis, and Transient T-DNA Expression Assays. *Arabidopsis* plants, ecotype Ws, were grown and maintained under standard growth conditions and were characterized for stable and transient *Agrobacterium*-mediated genetic transformation as described (22). Briefly, for tumor-formation assay, root segments from 10- to 14-day-old *in vitro*-grown *Arabidopsis* seedlings were infected with *Agrobacterium tumefaciens* A208, cultivated for 48 h at 25°C on hormone-free Murashige and Skoog (MS) medium and then washed and cultured for 4–5 weeks in hormone-free MS medium supplemented with timentin to kill the bacteria. Tumors were then counted and their morphology documented. For transient expression, root segments were infected with *Agrobacterium tumefaciens* EHA105 harboring a β -glucuronidase (GUS)-expressing binary vector and cultivated on hormone-free medium for 48 h, transferred to fresh medium for an additional 2 days, and stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronide (X-Gluc). For quantification

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Abbreviations: T-DNA, transferred DNA; ss, single stranded; ds, double stranded; T-strand, ss copy of T-DNA; Vir, virulence; DSB, double-strand break; NHEJ, nonhomologous end-joining; MS, Murashige and Skoog; GUS, β -glucuronidase.

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genetic complementation of the *ku80* mutant with its native gene and restoration of the complemented mutant's susceptibility to infection by a tumorigenic *Agrobacterium* strain (Table 1). This demonstrates that the *ku80* phenotype most likely results from disruption of the native *ku80* function by the mutation in the gene.

KU80-Overexpressing Transgenic *Arabidopsis* Plants Are Resistant to DNA Damage and Are More Susceptible to *Agrobacterium* Infection.

To further determine the molecular role played by KU80 in the transformation process, we produced transgenic *Arabidopsis* plants that overexpress the KU80-coding sequence under a 35S promoter. First, we evaluated the *in vivo* effect of KU80 overexpression on DNA repair by comparing the sensitivity of wild-type, KU80-overexpressing, and *ku80*-mutant plants to methyl methane sulfonate (MMS) (a DNA-damaging agent). Fig. 1*d* shows that, whereas the *ku80* mutant was hypersensitive, relative to controls, to 100-ppm MMS, KU80-overexpressing plants exhibited enhanced resistance to MMS, even at 150 ppm. Similarly, KU80-overexpressing plants exhibited enhanced resistance to bleomycin (another DNA-damaging agent). Thus, overexpression of KU80 in *Arabidopsis* plants can specifically enhance the plant's cellular resistance to DNA damage. Next, we performed both tumorigenesis and transient gene-expression assays (22) on wild-type and KU80-overexpressing plants. Because the *Arabidopsis* ecotype Ws is highly susceptible to *Agrobacterium* transformation, we altered the transformation assays as described (32) by using a 1,000-fold lower concentration of *Agrobacterium* inoculum to differentiate between wild-type and KU80-overexpressing plants. Fig. 1*e* shows that even at a low *Agrobacterium* concentration, infected roots from wild-type plants produced galls. However, the number of gall-producing roots was 2-fold higher with the KU80-overexpressing plants, and the average size of the transgenic galls was much larger on these roots, as compared with the wild type (Fig. 1*d* and Table 1). No differences in GUS expression level were observed between wild-type and KU80-overexpressing *Arabidopsis* plants (Table 1) after infection with the *uidA*-intron-carrying T-DNA. KU80-overexpressing plants were also more susceptible to infection with disarmed *Agrobacterium* carrying a T-DNA encoding a herbicide-resistance gene but exhibited a callus-regeneration response on nonselective media that was similar to that of wild-type plants (Table 2). Furthermore, the regenerated calli were similar in size and shape in wild-type and KU80-overexpressing plants, under both control and selective conditions. These results indicate that overexpression of KU80 does not affect transient gene expression from the T-DNA but increases the extent of T-DNA integration into the host genome, and that KU80 does not nonspecifically affect the plant's regeneration potential.

KU80 Binds to and Recombines dsT-DNA Molecules *in Planta*. Detection and repair of DNA damage in living cells begin with the recognition of DNA lesions by a KU protein complex. In addition, one line of experimental evidence suggests that dsT-DNA molecules, which are rapidly converted from T-strands to their ds form early in the infection process (33), are essential intermediates of T-DNA integration (reviewed in ref. 16). To test whether KU80 physically recognizes and interacts with dsT-DNA molecules *in planta*, we immunoprecipitated T-DNA molecules with KU80 from *Agrobacterium*-infected *Arabidopsis* cells, which were transiently expressing His-tagged KU80 (Fig. 2*a*). No PCR amplification of bacterial genes or the binary plasmid's backbone was observed in the various immunoprecipitation extracts (Fig. 2*a*), indicating the specific immunoprecipitation of dsT-DNA molecules by His-tagged KU80. Because KU80 binds to ds- but not ssDNA molecules during DSB repair (34), our results clearly indicate that dsT-DNA molecules are

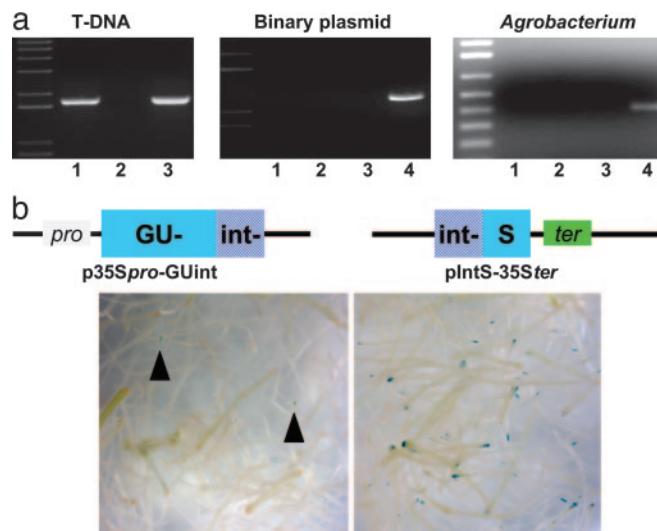


Fig. 2. The KU80 protein binds to and recombines dsT-DNA molecules *in planta*. (a) PCR analysis of T-DNA molecules immunoprecipitated and PCR-amplified as described (28) with antibodies against a His-tagged KU80 protein and using primers specific to T-DNA (Left), binary plasmid (center) and *Agrobacterium* VirE2 (Right). Lanes 1 (a and b) precipitated extract treated with anti-His antibodies; lanes 2 (a and b) mock-precipitated extract to which no antibody was added; lanes 3 (a and b) untreated total DNA extract; lane 4 (Center) amplification of plasmid DNA and lane 4 (Right) amplification of bacterial DNA. (b) T-DNA to T-DNA recombination assay. Wild-type (Left) and KU80-overexpressing (Right) root segments were cotransformed with two binary plasmids, each carrying a partial GUS expression cassette (i.e., promoter-GUSint and intGUS-terminator). T-DNA to T-DNA recombination and expression of the GUS reporter gene were monitored by 5-bromo-4-chloro-3-indolyl β -D-glucuronide (X-Gluc) staining. Arrows (Left) indicate the presence of GUS-stained tissue in wild-type root segments.

recognized as DSBs by the NHEJ repair machinery. It follows that KU80 should also be able to recognize the presence of two or even more T-DNA molecules and to facilitate their extra-chromosomal recombination to each other. To explore this possibility, we designed an assay that would enable us to monitor the extent of recombination events between two specific T-DNA molecules *in planta*. In this assay, we split a *uidA*-intron-expressing cassette into two parts and cloned each part on a different *Agrobacterium* binary plasmid (Fig. 2*b*). In the 35Spro-GUint binary plasmid, the C terminus of the *uidA* gene and part of the 173-bp intron were cloned under the control of the tandem 35S promoter, whereas in pIntS-35Ster, the N terminus of the *uidA* gene and part of the 173-bp intron were cloned downstream of the 35S polyA signal. Although functional expression of the *uidA*-reporter gene could not be achieved by transfection of plant cells with either plasmid alone (data not shown), reconstruction of the *uidA*-intron expression cassette by directional recombination of two T-DNA molecules in plant cells was expected to result in functional GUS expression. After infection with a mix of the two *Agrobacterium* strains, functional GUS expression was indeed observed in wild-type *Arabidopsis* roots (Fig. 2*b*). However, events of T-DNA to T-DNA recombination leading to GUS expression in wild-type plants were rare (an average of four events per 150 root segments) as compared with KU80-overexpressing plants (an average of 40 events per 150 root segments) thus indicating the crucial role of KU80 in recognizing T-DNA molecules *in planta*.

KU80 Increases the Rate of T-DNA Integration. The efficiency of KU80 binding to dsT-DNA molecules in transgenic plants is expected to affect the overall efficiency of T-DNA integration into the plant genome, even without applying selection pressure.

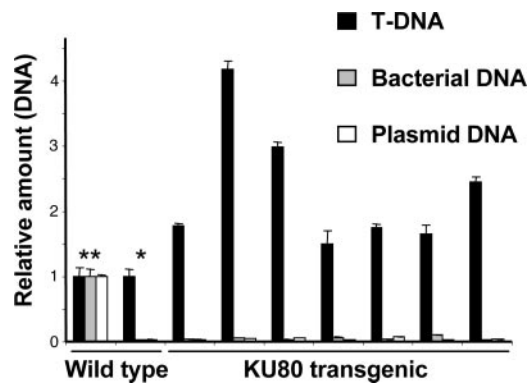


Fig. 3. Quantitative real-time PCR analysis of T-DNA integration in wild-type and KU80-transgenic plants. The amounts of integrating T-DNA molecules (black), bacterial DNA (gray), and binary plasmid DNA (white) were normalized against actin, serving as an internal control. The amounts of T-DNA molecules from individual KU80-overexpressing calli are presented in relative amounts in comparison to an average T-DNA amount from five individual wild-type calli. The amounts of bacterial and plasmid DNA molecules amplified from wild-type and KU80-overexpressing calli (*) are presented in relative amounts in comparison with control experiments, where bacterial and plasmid DNA was added to wild-type calli (**). Each reaction was repeated three times.

To study this question, we used real-time PCR to quantify the rate of T-DNA integration after infection of transgenic and wild-type *Arabidopsis* roots and found the number of T-DNA integration events in KU80-transgenic plants to be 1.5- to 4.1-fold higher than in wild type (Fig. 3). PCR amplification of bacterial genes and binary plasmid backbone could be observed in samples where bacterial or plasmid DNA had been intentionally added but not in wild-type or KU80-transgenic clusters, indicating the specific amplification of integrated T-DNA molecules (Fig. 3).

Discussion

Agrobacterium is a unique organism capable of genetically transforming many eukaryotic cells. To achieve its goal of transferring its T-DNA molecule and integrating it into the host cell genome, *Agrobacterium* relies not only on a set of its virulence proteins but also on their cellular partners and on various basic cellular processes (reviewed in refs. 2 and 8). For example, dynein-motor-assisted movement on microtubules has been proposed to be the driving force behind T-DNA transport through the host cell cytoplasm (35), and the interactions of the VirE2 and VirD2 proteins with the host factors VIP1 (11) and importin α (12) have been found to be important for T-complex import into the host cell nucleus (36). Inside the nucleus, T-DNA interactions with several host proteins have been suggested to facilitate its movement to points of integration (37, 38) and its dissociation from its escorting proteins (13). The last steps of the transformation process, i.e., conversion of the T-DNA to a ds molecule and its integration into the host cell genome, are most likely effected exclusively by host proteins and the host cell DNA-repair machinery (16). Indeed, genetic analysis has revealed the importance of various DNA-repair proteins for T-DNA integration in yeast cells (17, 18), and the plant's DNA-packaging protein H2A has been found essential for T-DNA integration in plants (32). Yet the key DNA-repair proteins and the molecular mechanism for T-DNA integration in plants has remained mostly unknown. Our results provide molecular evidence for the function of the plant protein KU80, a key protein in the NHEJ pathway, in T-DNA integration, suggesting that KU80 serves as the first point of contact between the T-DNA and the DNA-repair machinery. Many *Arabidopsis*

mutants have been reported to be blocked in the last steps of the transformation process (22). However, although some of them may be blocked in the integration step itself (i.e., ref. 32), others may be deficient in various cellular processes leading to the formation of gall tissue, such as T-DNA expression, dedifferentiation of the transformed cells and redifferentiation into gall tissue. That an *Arabidopsis ku80* mutant was blocked in one of the last steps of the genetic transformation process and that T-DNA nuclear import, conversion to the ds form and transient expression were not affected by the lack of KU80 protein support the notion that *ku80* is not defective in T-DNA expression but most probably in T-DNA integration. Furthermore, that callus regeneration from *ku80*-mutant and KU80-overexpressing plants was similar to that from wild-type plants supports the notion that *ku80* is not affected in dedifferentiation or redifferentiation of transformed or wild-type cells. KU80 overexpression resulted in larger tumors, but not callus clusters, after infection with wild-type or disarmed *Agrobacterium*, respectively. The herbicide-resistant callus clusters obtained from wild-type and KU80-overexpressing plants were similar in size, and the number of T-DNA integration events was higher in KU80-overexpressing plants. This suggests that the increased tumor size can be attributed to multiple integration events in the transformed tissues. The latter would lead to a larger number of oncogenic genes, and their expression would most likely result in higher cell division and proliferation rate. Interestingly, similar to many other *rat* mutants (31), the *ku80* mutant can be transformed by *Agrobacterium*-mediated transformation via flower dip (20, 21). Because of the nature of the flower-dip transformation method, which is performed under relatively uncontrolled conditions, it is rather difficult to use it to clearly define the susceptibility or resistance of mutant lines; it can, however, serve to determine whether they are transformable or not. Indeed, controversy over the role of KU80 in flower-dip transformation still exists (20, 21), and additional data are required to clearly determine whether KU80 is essential during ovule transformation as it is in somatic cells. Nevertheless, it is likely that different and perhaps supplemental pathways govern the repair of DNA breaks in somatic and germ-line tissues; more data are required to accurately compare the mechanism of integration in these different tissues.

Previous studies have shown that T-DNA molecules, arriving from a single or two different *agrobacteria*, can integrate into the same location on the plant chromosome (39–42). Sequence analysis of some insertions has revealed precise fusion between two right-border ends, an event that can occur only through integration of dsT-DNA intermediates. Furthermore, one line of experimental evidence suggests that dsT-DNA molecules, which are rapidly converted from T-strands to their ds form early in the infection process (33), are dominant intermediates of T-DNA integration (14–16, 43) and do not necessarily represent a minor pathway of the integration process. These studies have also shown that dsT-DNA molecules can be directed to DSBs (14, 15, 43). Detection and repair of DNA damage in living cells begin with the recognition of DNA lesions by a protein sensor complex and are followed by its repair by DNA-repair proteins (30). More specifically, a simple DNA DSB is first recognized by a KU protein complex and then directed for repair by the XRCC4–LIG4 complex (44). Immunoprecipitation of dsT-DNA molecules with KU80 from infected plants and the high number of T-DNA to T-DNA recombination events in KU80-overexpressing plants clearly suggest that KU80 physically recognizes and interacts with dsT-DNA molecules, recognizing them as DSBs, and leads them to repair by the NHEJ repair machinery. This therefore suggests a simple DNA-repair mechanism for T-DNA integration into the genome.

Interestingly, the efficiency of T-DNA integration in KU80-transgenic plants was lower than expected when comparing the efficiency of extrachromosomal T-DNA to T-DNA recombina-

tion in KU80-transgenic and wild-type plants. Because naturally occurring DSBs may act as molecular “baits” for the incoming T-DNA molecules (14), and because their extent is most likely similar in wild-type and transgenic plants, it would be rational to assume that KU80 overexpression in transgenic plants leads to more efficient DNA-repair activity and to an overall reduction in DSB sites accessible for T-DNA integration. Thus, even a 1.5-fold increase in T-DNA integration rate in KU80-transgenic plants is, in fact, significant and further supports the crucial and direct participation of KU80 in the integration process.

Our data provide a mechanistic basis for the role of NHEJ in the integration process and suggest that dsT-DNA molecules are

recognized as DSBs by the host’s NHEJ machinery. We identified KU80 as a key protein in the T-DNA integration process and suggested that it recognizes and binds to dsT-DNA intermediates. Other DNA-repair proteins, such as KU70, RAD50, and MRE11, have been found to be important for T-DNA integration in yeast cells by NHEJ (17, 18), and it is crucial to study the function of their plant orthologues to further understand the mechanism of T-DNA integration in plant species.

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