Role of non-homologous end-joining in T-DNA integration in Arabidopsis thaliana

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Chapter 4

Mre11 and Ku80 control different pathways of DNA repair and T-DNA integration in Arabidopsis

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Abstract

The Mre11 complex has a critical role in DNA damage signaling and DSB repair. The Arabidopsis Mre11 protein shares a highly conserved N-terminus with yeast and mammalian Mre11. T-DNA insertions in this conserved region, thus fully inactivating the gene, resulted in many developmental defects including sterility (*mre11-1*, *mre11-3*). The *mre11-4* mutant with T-DNA insertion in the C-terminal region exhibited a similar phenotype, but the *mre11-2* mutant with a slightly downstream insertion was fully fertile and grew normally. Nevertheless, also this latter mutant was hypersensitive to DNA damaging agents, indicating defective DNA repair. By yeast two-hybrid assays, we found that the Mre11-2 protein (truncated at amino acids 1 to 529) still interacted with Rad50, but the Mre11-4 protein (truncated at amino acids 1 to 499) did not, indicating that the area between amino acid 499 and 529 is important for interaction with Rad50. As many functions of Mre11 rely on complex formation with Rad50, this explains the large difference between *mre11-2* and *mre11-4* mutants. As the *mre11-2* mutant showed sensitivity to DNA damage, we tested whether this was due to a defect in c-NHEJ repair by generating *ku80mre11-2* double mutant plants. Interestingly, the *ku80mre11-2* double mutant plants were more sensitive to DNA damage stress than single mutant plants and moreover were resistant to *Agrobacterium*-mediated transformation. These results indicate that Mre11 is important for backup NHEJ pathways involved in T-DNA integration.

Introduction

Non-homologous end-joining (NHEJ) is believed to be the major pathway for the repair of DSBs in most higher eukaryote cells. The classical NHEJ (c-NHEJ) pathway embraces as critical factors Ku70/80 and Lig4, which are also found in plant cells. In the absence of Ku, efficient repair still occurs indicating that the cell has backup pathways for repair, which are called backup NHEJ (b-NHEJ) or sometimes also microhomology-mediated end-joining (MMEJ) or alternative NHEJ (a-NHEJ). Such repair pathways were discovered in yeast (*Saccharomyces cerevisiae*) (Boulton and Jackson 1996) and in mammalian cells (see review Deriano and Roth 2013). The c-NHEJ pathway is relatively precise but still sometimes causes small deletions or insertions. In the absence of Ku, resection of the ends becomes possible, which may lead to large deletions, insertions and translocation which are features of b-NHEJ. However, the molecular mechanism underlying b-NHEJ remain unclear. Also in plants, evidence was obtained that repair can occur by such backup pathways to maintain plant genome integrity (Charbonnel *et al.* 2010; Jia *et al.* 2013). Several factors that are involved in b-NHEJ have already been identified: PARP1/2, Ligase3, XRCC1, Pol θ, CtIP and Mre11.

Mre11 has been well defined in DNA damage response (DDR) and homologous recombination (HR). It combines with Rad50 and Nbs1 (Xrs2 in yeast) to form the MRN (MRX in yeast) complex and acts as a DSB sensor for activation of ATM and downstream signaling. Mre11 has both single-stranded DNA endonuclease activity and 3' to 5' exonuclease activity, and thus MRN plays a crucial role in promoting DSB end resection, which is likely to determine pathway choice between HR and NHEJ and repair outcome (see review Ceccaldi *et al.* 2016). However, the MRN/MRX complex, and Mre11 in particular, have been found to have also a direct role in NHEJ repair pathways. In yeast, MRX has been implicated in NHEJ repair and in the MMEJ pathway (Chen *et al.* 2001; Ma *et al.* 2003). Deficiency of the MRX complex resulted in a significant reduction in the frequency of T-DNA integration by NHEJ in yeast (van Attikum *et al.* 2001). In mammalian cells, Mre11 promotes both c-NHEJ and b-NHEJ pathways during the repair of I-SceI endonuclease-induced DSBs (Rass *et al.* 2009; Xie *et al.* 2009).

In plants, it has been shown that MRN mutants (Mre11, Rad50 and Nbs1) exhibit an increased sensitivity to DNA damage (Gallego *et al.* 2001; Bundock and Hooykaas 2002; Waterworth *et al.* 2007). Four *Arabidopsis thaliana* T-DNA insertion mutant lines of the *MRE11* gene were previously described: *mre11-1* and *mre11-2* (Bundock and Hooykaas 2002), *mre11-3* (Puizina *et al.* 2004), *mre11-4* (Šamanić *et al.* 2013). The *mre11-2* plants displayed normal growth and fertility, while *mre11-1, mre11-3* and *mre11-4* mutant lines had many developmental defects and were sterile. These reports demonstrated that Mre11 has essential roles in the response to irradiation-induced DSBs, meiotic recombination and maintenance of chromosomal stability.

In this chapter, we compared *mre11-4* and *mre11-2* both with insertions in the C-terminal part of Mre11 to determine which function is missing in *mre11-4* versus *mre11-2*. The yeast two-hybrid results showed that *mre11-2* still preserved interaction with Rad50, but *mre11-4* did not. In order to study the role of Mre11 in plant cells, including NHEJ and *Agrobacterium* T-DNA integration, we generated a homozygous double mutant *ku80mre11-2,* in which the c-NHEJ repair pathway is inactivated. More sensitivity to DNA damage stress

was found in this double mutant revealing a crucial role for Mre11 in DNA repair in absence of Ku80. Using yeast as a model it was shown in our lab that T-DNA integration in yeast is dependent on the c-NHEJ pathway of DSB repair (van Attikum et al. 2001). However, inactivation of c-NHEJ or of both c-NHEJ and factors supposedly involved in b-NHEJ pathways in plants (PARP1, PARP2, XRCC1) still allowed T-DNA integration by *Agrobacterium* (see Chapter 2), suggesting that there must be other important proteins and/or another NHEJ pathway mediating the T-DNA integration in plant cells. Remarkably, the *ku80mre11-2* double mutant was resistant to T-DNA integration, while the single mutants still were proficient in T-DNA integration. This suggests that Ku80 and Mre11 control different pathways of T-DNA integration, each of which functions independently in *Agrobacterium* T-DNA integration in plant cells as only in the absence of both of them integration of T-DNA was not observed by the root transformation assay.

Materials and methods

Plant material

The T-DNA insertional *Arabidopsis thaliana* mutants of *ku80* and *mre11-2*, have been described previously (Bundock and Hooykaas 2002; Jia *et al.* 2012). The double *ku80mre11-2* mutant was produced by crossing *ku80* and *mre11-2* homozygous mutants followed by PCR analysis of progeny for identification of double homozygous plants.

Assays for sensitivity to bleomycin and methyl methane sulfonate (MMS)

Seeds of wild type, *ku80, mre11-2 and ku80mre11-2* mutants were surface-sterilized as described (Weijers *et al.* 2001) and germinated on solid ½ MS medium without additions or containing 0.02 μg/ml or 0.05 μg/ml bleomycin (Sigma), 0.005% (v/v), 0.007% or 0.01% MMS (Sigma) and photographed after 2 weeks. After 10 days growth, the fresh weight (compared with controls) was determined by weighing the seedlings in batches of 20 in duplicate. Statistical analyses were performed using Prism version 5 (GraphPad Software Inc.).

Cell Death Assay

Four days old seedlings on MS-agar plates were transferred to new MS-plates with DNA damaging agents. After one or four days treatments, 20 seedlings were placed in propidium iodide solution (PI, Sigma-Aldrich, 5 μg/ml in water) for 1 min and rinsed three times with water. Root tips were then transferred to slides in a drop of water and covered with a cover slip for observation under the fluorescence microscope.

Yeast two-hybrid Assays

Full-length Mre11 was cloned into pACT2, while full length Rad50 was cloned into pAS2.1 (James *et al.* 1996). $Mre11_{(1\text{-}499)}$, $Mre11_{(1\text{-}510)}$, $Mre11_{(1\text{-}529)}$, $Mre11_{(530\text{-}720)}$ were amplified by PCR and cloned into pACT2 and pAS2.1. Primer sequences are presented in Table1. All PCR fragments were verified by sequencing. Interaction assays were performed by co-transformation of bait and prey plasmids into yeast strain PJ69-4A as previously described (Gietz et al., 1992), and plated on MY minimal medium, supplemented with methionine, uracil, adenine and histidine and lacking leucine and tryptophan (MY/–LW). Subsequently,

cells were incubated for 16 h in liquid MY/–LW and spotted onto selective solid MY medium lacking leucine, tryptophan and histidine (MY/–LWH) supplemented with increasing concentrations of 3-amino-1,2,4-triazole (3-AT, Sigma) ranging from 0 to 30 mM. Yeast cells were allowed to grow for 5 days at 30°C.

Root transformation

Root transformation was performed as described (Vergunst *et al.* 2000). Root segments were infected with *Agrobacterium* LBA1100 (pCambia3301). After co-cultivation on callus induction medium containing 100 μM acetosyringone for 48 hours, root segments were washed, dried and incubated on shoot induction medium supplemented with 30 μg/ml phosphinothricin, 500 μg/ml carbenicillin and 100 μg/ml vancomycin. After 3-4 weeks, plates were photographed and transformation efficiencies were scored as infected root segments that produced any form of green callus. Statistical analyses were performed using Prism version 5 (GraphPad Software Inc.).

Results

The C-terminal part of Mre11 contains the domain interacting with Rad50

The Arabidopsis Mre11 protein shared a large conserved domain in the N-terminus with Mre11 from other organisms, but there was less clear homology in the C-terminus (**Figure 1A**). Four Arabidopsis *MRE11* T-DNA insertion mutants have been characterized. The *mre11-2* mutant exhibited a normal growth phenotype and was fertile, whereas the *mre11-1*, *mre11-3* and *mre11-4* mutants were sterile and showed obvious morphological abnormalities. While the T-DNA insertions in *mre11-1* and *mre11-3* were localized in the N-terminal part of the gene, the insertions in *mre11-2* and *mre11-4* were located in the C-terminus. As a result of T-DNA insertion, 10 additional amino acids (NTQLKNVNMI) may form the C-terminus of the predicted protein in the *mre11-2* mutant, while 35 additional amino acids (ARRYRFSCLITFFN-SGLLFQTGTTLNPFSGYSFDL) are predicted at the C-terminus in the *mre11-4* mutant. Except for the extra amino acids formed at the C-terminus, putative truncated Mre11 proteins formed in the *mre11-2* and *mre11-4* mutants would differ only by 30 amino acids. *In silico* analysis of the predicted truncated Mre11 proteins showed that the *mre11-4* deletion removes a large region that is highly conserved in Mre11 proteins of different plants, while only a small part of this is deleted in the Mre11 protein encoded by the *mre11-2* allele (**Figure 1B**).

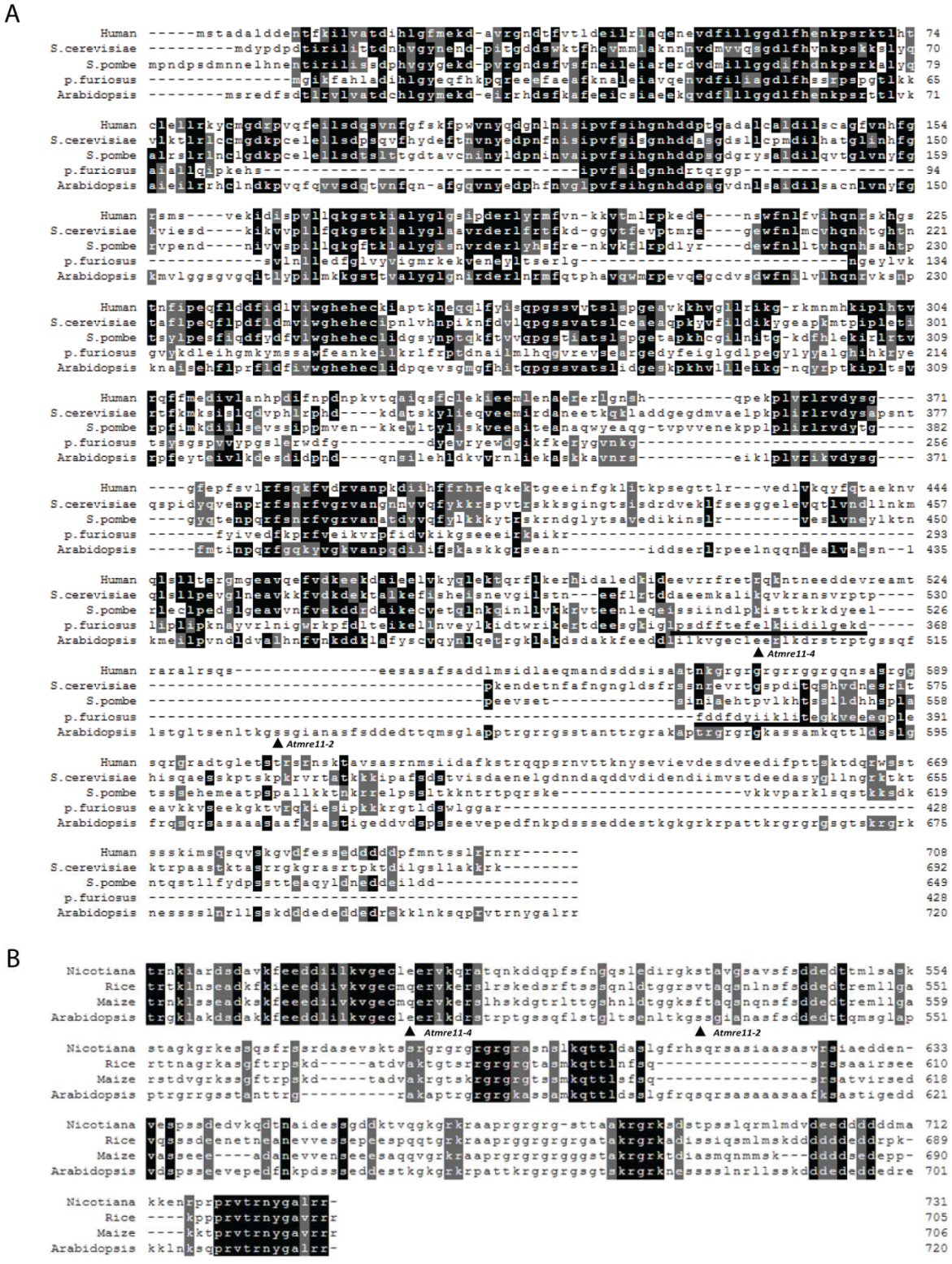

Figure 1. (**A**) Alignment of Mre11 proteins from *Homo sapiens*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pyrococcus furiosus* and *Arabidopsis thaliana*. The conserved amino acids are indicated with black and gray boxes. The Rad50 binding domains (RBD) in *P. furiosus* are underlined. (**B**) Alignment of Mre11 C-terminus from *Nicotiana tabacum*, *Oryza sativa* (rice), *Zea mays* (maize) and *Arabidopsis thaliana*.

To get a better understanding of the functional differences between the Mre11-2 protein truncated at amino acid 529 and the Mre11-4 protein truncated at amino acid 499, yeast two hybrid assays were performed (**Figure 2**). Full-length Mre11 displayed a strong interaction with Rad50 and with itself, indicating that the Arabidopsis Mre11 can potentially homodimerize and form a Mre11-Rad50 complex as expected. To test if the truncated proteins formed in *mre11* mutants still could interact with Rad50 and homodimerize, each of four fragments of Mre11 (amino acids 1 to 529; 1 to 510; 1 to 499; 530 to 720) cloned in pAS2-1 and pACT2 were cotransformed into yeast. A full-length clone of Rad50 in pACT2 and each of four fragments of Mre11 cloned in pAS2-1 were cotransformed into yeast. We found that amino acids 1 to 499 of Mre11 were sufficient for self-interaction. Although Mre11 amino acids 1 to 529 displayed a strong interaction with Rad50 comparable to that observed for full-length Mre11, the smaller version (amino acids 1 to 499) did no longer interact with Rad50. Apparently, presence of the area from amino acids 499 to 529 overlapping with a region that is strongly conserved in plant Mre11 is essential for the interaction of Mre11 with Rad50. Indeed, an Mre11 construct (amino acids 1 to 510) preserving this conserved region, but only 11 amino acids larger than the Mre11-4 protein Mre11 (amino acids 1 to 499) was now able to interact with Rad50. The C-terminal part of Mre11 (aa 530 to 720) by itself did neither homodimerize nor interact with Rad50. These results revealed that the ability to form of Mre11 homodimers and Mre11-Rad50 complexes are preserved in the Mre11-2 protein, but that the Mre11-4 protein could no longer form a complex with Rad50. As Mre11 relies for most of its functions on the formation of a complex with Rad50 (and Nbs1), this explains the different phenotypes of the *mre11-2* mutant in comparison with *mre11-4* and the other T-DNA insertion mutants.

We further tested the interaction between Mre11 and Nbs1 which is the third component of the MRN complex. Although such interactions between plant Mre11 and Nbs1 were observed previously (Waterworth *et al.* 2007), no interaction was observed by our two-hybrid analysis. This may be due to the different yeast two-hybrid systems used. As it was been reported that Mre11 plays a role in b-NHEJ pathways, we studied whether Mre11 interacts with proteins known to be involved in the b-NHEJ repair pathway (such as PARP1, PARP2, LIG1, XRCC1, COM1 and RAD9) by two-hybrid analysis as well. However, no interaction between Mre11 and any of those proteins could be detected.

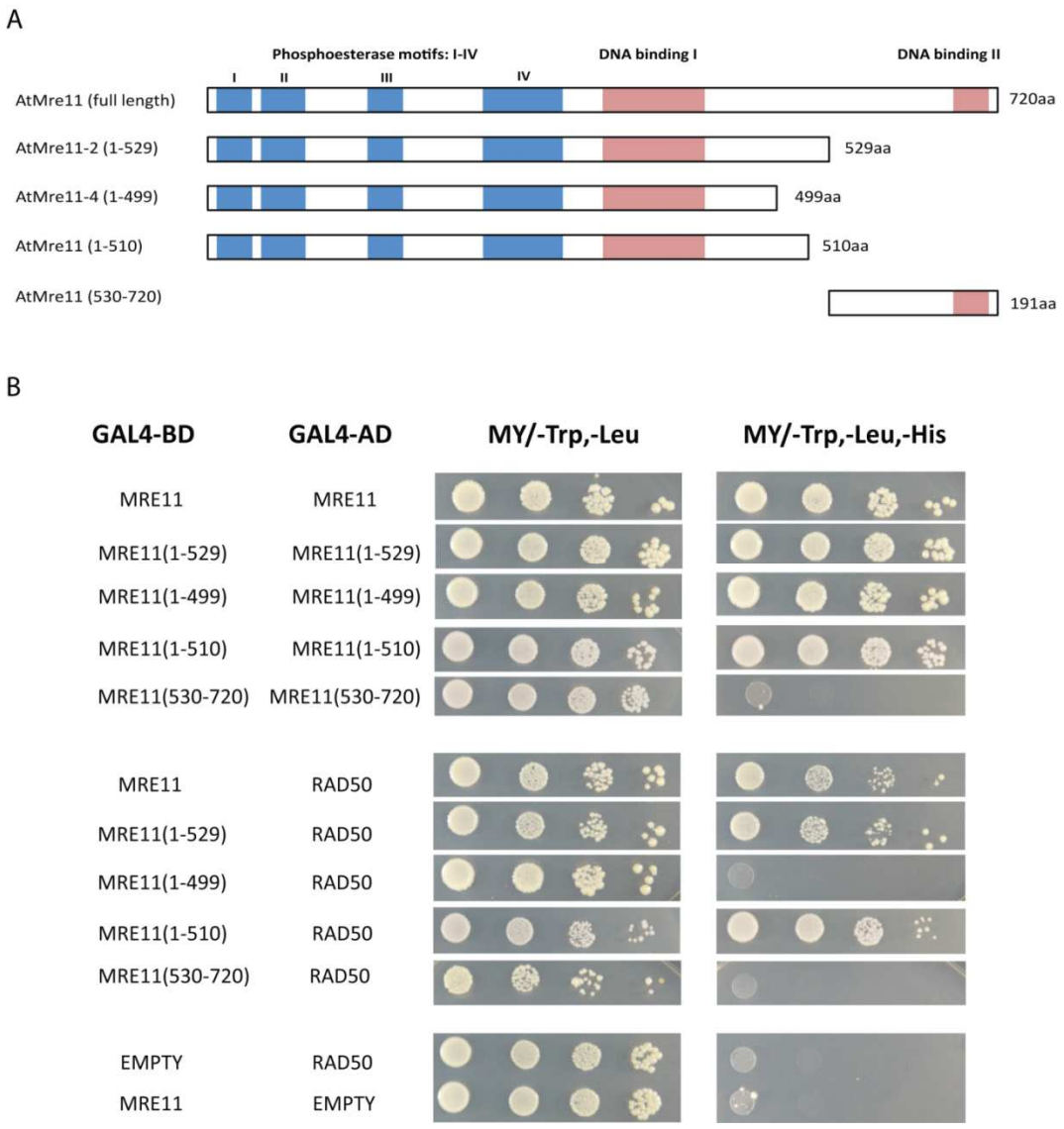

Figure 2. Yeast two-hybrid analysis of MRE11 interactions. (**A**) Schematic regions of MRE11 analyzed in the *in vitro* interaction studies and the constructs used in the yeast two-hybrid analysis. (**B**) Interaction of MRE11 full length protein and deletions with RAD50 protein. The interactions between MRE11 and RAD50 or MRE11 itself resulting in histidine autotrophy.

The Ku80mre11-2 double mutant is more sensitive to DNA damage stress than each of the single mutants

Previously, in our lab it was shown that the *ku80* and the *mre11-2* single mutants were hypersensitive to DNA damage stress (Bundock and Hooykaas 2002; Jia *et al.* 2013). In order to test whether the mre11-2 mutation affects the same or a different repair pathway as Ku80, the *ku80mre11-2* double mutant was obtained by crossing the single mutants and tested for sensitivity to genotoxic agents (**Figure 3A**). Without any treatments, the double mutant grew similarly as the wild type. However, upon treatment with bleomycin or MMS, the double mutant grew more slowly and developed fewer true leaves in the assay period than each of the single mutants, indicating increased DNA damage sensitivity in double mutant plants. We also evaluated the effect of DNA damage on the growth of 10-days-old plate-grown plants by

quantification of the fresh weight (**Figure 3B**). We found that the fresh weight of double mutant plants was dramatically reduced in comparison with that of wild type and each of the single mutants, when treated with bleomycin or MMS.

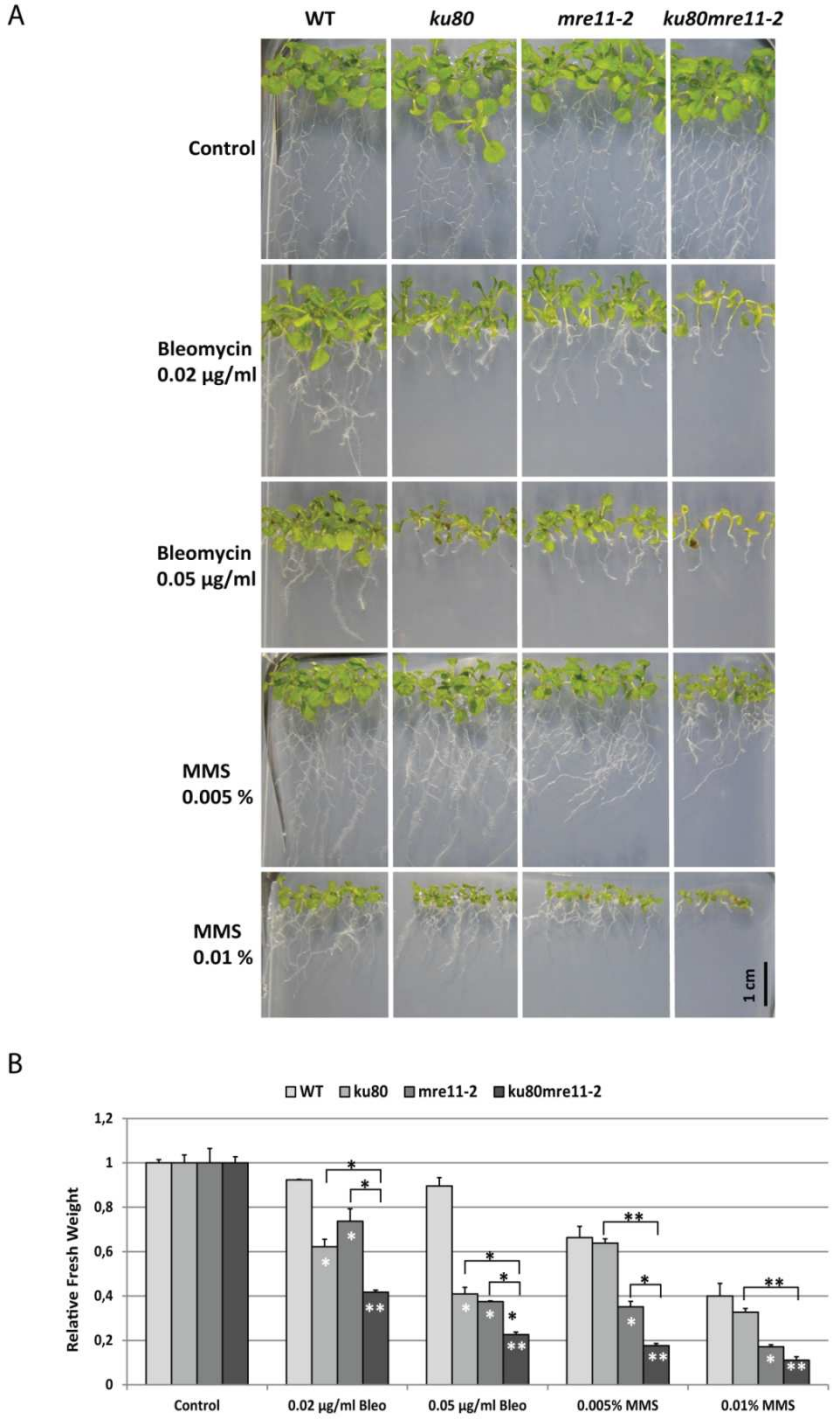

Figure 3. The *ku80mre11-2* double mutant is hypersensitive to DNA damaging treatments. (**A**) Phenotypes of wild-type plants and *ku80*, *mre11-2* and *ku80mre11-2* double mutants germinated on ½ MS medium (control) or ½ MS medium containing 0.02, 0.05µg/ml bleomycin or 0.005%, 0.01% MMS photographed 2 weeks after germination. (**B**) Fresh weight of 10-days-old wild-type and *ku80*, *mre11-2* and *ku80mre11-2* double mutants treated with 0.02, 0.05 µg/ml bleomycin or 0.005%, 0.01% MMS. For each treatment 20 seedlings were weighed in duplicate. Fresh weight of the plants grown 10 days without bleomycin or MMS was set on 1. Error bar represent SD. *Anova* test; for *, $p<0.001$; **, $p<0.0001$.

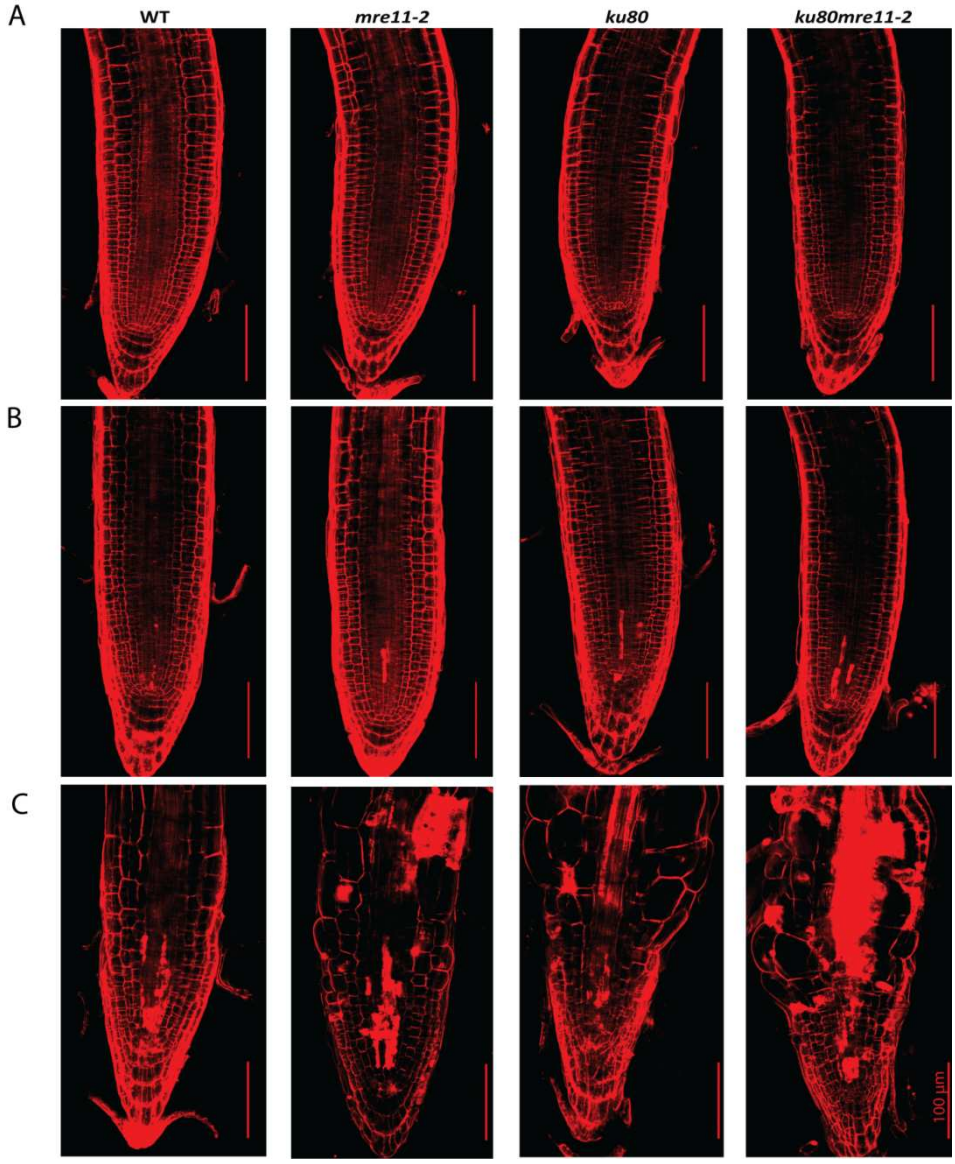

Figure 4. Cell death profile in root tips of wild type and mutants. PI-staining of root tips of 4-days-old wild-type, *ku80*, *mre11-2* and *ku80mre11-2* seedlings grown on MS medium and transferred to MS medium with 0.05 µg/ml bleomycin for 1 day (**B**) and 4 days (**C**), and to MS medium without bleomycin for 2 days as control (**A**).

As the *ku80mre11-2* double mutant plants might have difficulty in repairing DNA damage, this might lead to more cell death than in the wild type or each of the single mutants. To test this, we used propidium iodide (PI) staining to reveal cell death. As can be seen in **Figure 4**, no cell death was observed in wild type and mutants under the normal growth conditions. Bleomycin treatment caused cell death in the root meristem of both wild type and the mutants, and this increased over time. More cell death was seen in the roots of the double mutant than in that of the single mutants, which were more damages than the wild type (**Figure 4B**). When the time period of the genotoxic stress was extended, a much shorter meristematic zone was observed in the root tip of the double mutant (**Figure 4C**), which might be due to the enhanced cell death in the root meristem. Meanwhile, the epidermal cells

of the double mutant were also more deformed and enlarged than those of wild type and each of the single mutants (**Figure 4C**).

Taken together, the results showed that *ku80mre11* double mutant plants grow similarly as wild type plants under normal growth conditions, but they are more sensitive to DNA damage and grow slower in the presence of genotoxic agents than each of the single mutants. This indicates that Mre11 probably plays an important role in Ku-independent DNA repair pathways in somatic plant cells.

The Ku80mre11-2 double mutant is resistant to Agrobacterium mediated T-DNA integration

Previously, we found that both the *ku80* and *mre11-2* mutants were somewhat recalcitrant to transformation in the floral dip procedure (Jia *et al.* 2012), but other groups have shown that the *ku80* mutant is either not or little affected in floral dip or root transformation by *Agrobacterium* (Gallego *et al.* 2003; Park *et al.* 2015). In order to test whether the *ku80mre11-2* double mutant is affected more strongly in *Agrobacterium*-mediated T-DNA transformation, we used the *ku80mre11-2* double mutant together with wild type and single mutants in the root transformation procedure (**Figure 5**). To this end, roots were cocultivated with an *Agrobacterium* strain containing the pCambia3301 binary vector, and callus formation was selected on medium with phosphinothricin. The transformation frequencies of the single mutants were not significantly different from the wild type. The calli formed on the roots of the *mre11-2* mutant grew somewhat greener than those formed by the wild type. This was due to a difference in ecotype between the *mre11-2* mutant (ecotype Ws) and the *ku80* mutant (ecotype Col), but did not influence the transformation results. While wild type and single mutants were transformed at a similar frequency, surprisingly, the *ku80mre11-2* double mutant only produced very few green calli in the transformation procedure (**Figure 5A**). Upon longer incubation of these calli on selection plates no green shoots were formed and the calli eventually died (**Figure 5B**, **6A**), indicating that the *ku80mre11-2* double mutant cannot be transformed by root transformation. One allele of Ku80 was sufficient for rescue as can be seen by the efficient transformation of roots with the ku80+/-mre11-2-/- genotype (Figure 5A). Also, calli and green shoots were formed normally on medium without selection, although calli of the double mutant grew a bit slower than those of wild type and single mutants (**Figure 6B**).

In order to test whether the lack of callus formation was due to the lack of T-DNA transfer or a deficiency in T-DNA integration, roots were transformed by an Agrobacterium strain with the pCambia3301 binary vector. Plant cells receiving T-DNA from this vector express the *GUS* gene before integration, which disappears in due time when the T-DNA is not stably integrated. The results from such transient GUS assay showed that transient transformation (and thus T-DNA transfer) is not affected in these mutants (**Figure 5C**).

Taken together, these results thus show that inactivating both Ku80 and Mre11 at the same time leads to resistance to *Agrobacterium*-mediated stable T-DNA transformation, but does not negatively affect T-DNA transfer. This suggests that Ku80 and Mre11 are involved in two different pathways of T-DNA integration, each of which is not essential, but after inactivation of both transformation is reduced to below detection level.

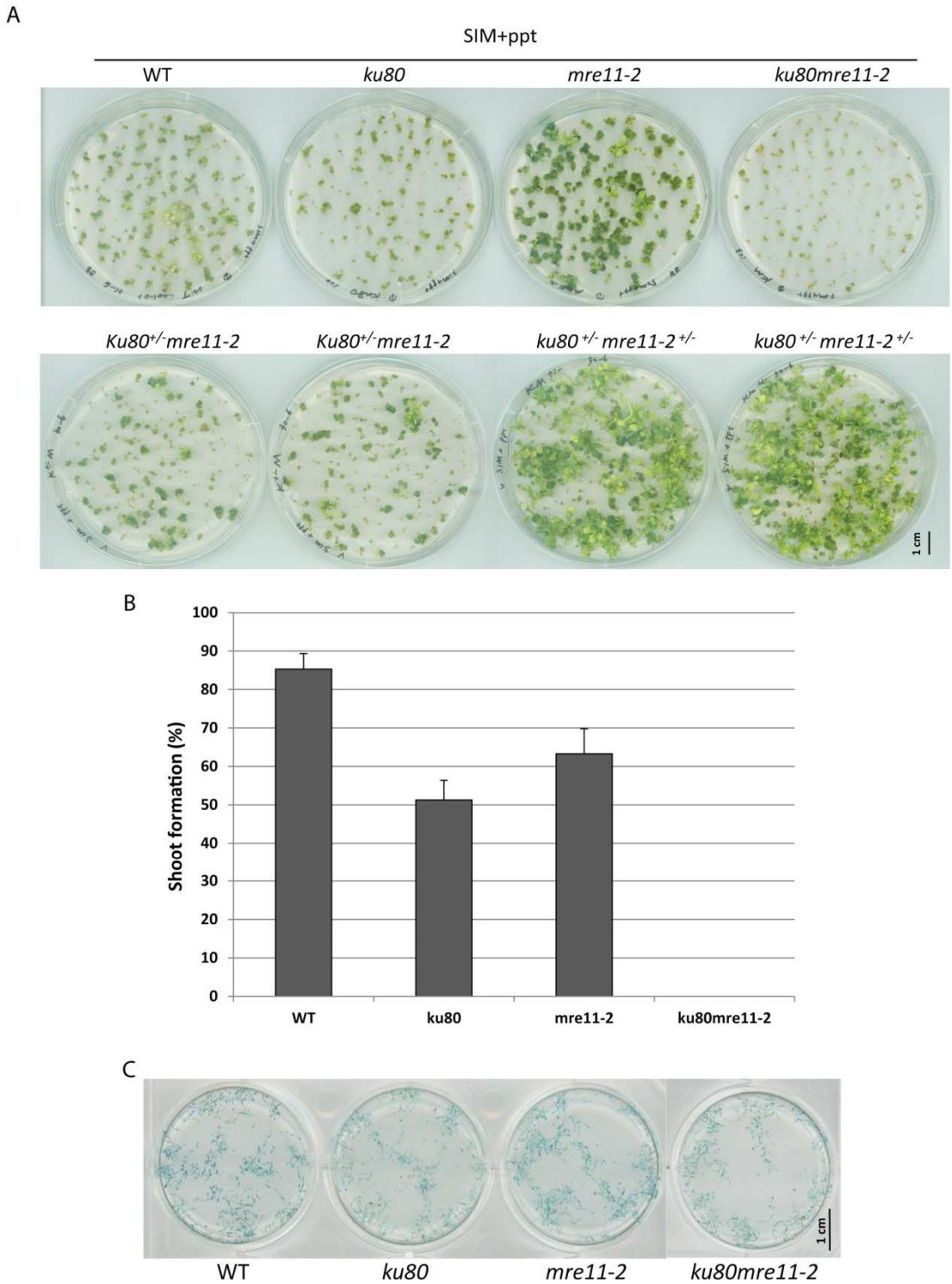

Figure 5. Root transformations of mutants. **(A)** Root segments from wild-type and mutant plants were co-cultivated with *Agrobacterium* strain LBA1100 (pCambia3301) for 48 hours, and transferred to shoot induction medium (SIM) with phosphinothricin selection (ppt). Photographs were taken 4 weeks after cocultivation. **(B)** shoot formation rate, represented by the percentage of green calli with shoot after 6 weeks cultivation on selection plates. **(C)** Root segments from wild-type and mutant plants were stained with X-Gluc overnight after 72 hours cocultivation.

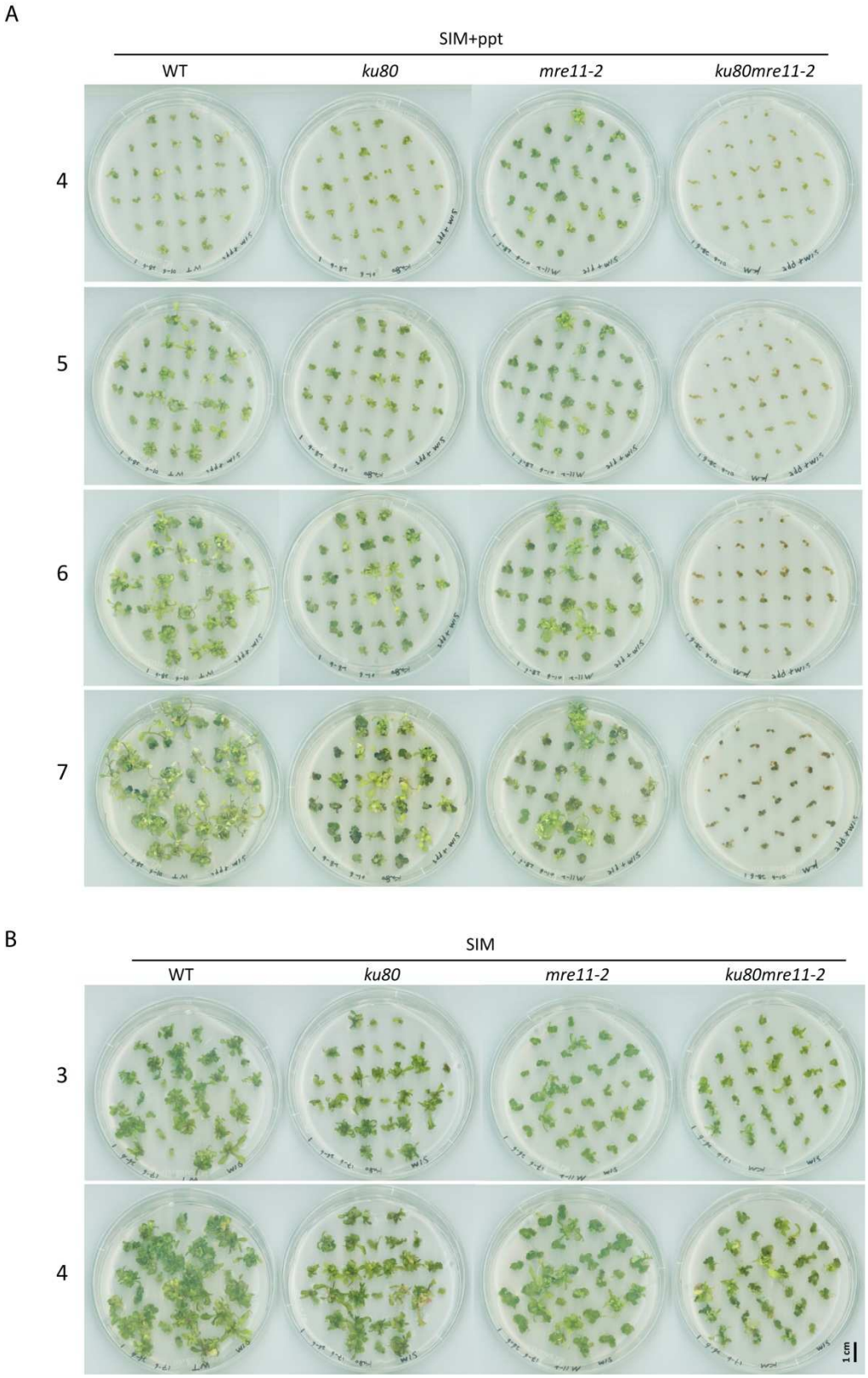

Figure 6. Shoot development on calli from root transformations. (**A**) After 3 weeks growth on shoot induction medium (SIM) with phosphinothricin (ppt) selection, green calli were transferred to fresh shoot induction medium with phosphinothricin. (**B**) After cocultivation with *Agrobacterium*, root segments were transferred to shoot induction medium without selection. After 3 weeks green calli were transferred to fresh shoot induction medium plates without selection. Photographs were taken every week after transfer to fresh plates. The number of 3, 4, 5, 6, 7 indicates the weeks of cultivation.

Discussion

The predicted protein sequence of the Arabidopsis Mre11 orthologue contains a highly conserved N-terminal nuclease domain which is essential for repair in yeast and human, but a less conserved region in the Mre11 C-terminus. Three different T-DNA insertion lines of Arabidopsis *MRE11* have many developmental defects and are sterile suggesting that the functions of Arabidopsis Mre11 are severely compromised in these mutants. The *mre11-2* mutant, however, is fertile and grows normally. The T-DNA insertions in the *mre11-4* and *mre11-2* mutant both leave the N-terminal nuclease domain of the encoded Mre11 protein intact. Our results from two-hybrid analysis demonstrated that the interaction domain necessary for the formation of a Mre11-Rad50 complex is still present in the Mre11-2 protein, but absent in Mre11-4. Mre11 relies for most of its functions on the formation of a Mre11-Rad50-Nbs1 complex explaining the phenotypic differences between the two mutants (Stracker and Petrini 2011). Rad50 binding domains (RBD) have been identified in the C-terminal part of *Pyrococcus.furiosus* Mre11 (Williams *et al.* 2011). Although this C-terminal region is less conserved, the C-terminal region of Mre11 in yeast and humans also have been indicated to be involved in the interaction with Rad50 (Chamankhah and Xiao 1999; Park *et al.* 2011). Our studies also point out that a region in the Arabidopsis Mre11 C-terminus likewise participates in Rad50 binding. Thus, these regions may form a similar three dimensional structure as the RBD of *P.furiosus* Mre11, although they share a low conservation in primary structure.

The *mre11-2* mutant preserves the Rad50 binding activities, grows normally but still shows hypersensitivity to DNA damaging agents. This suggests that either the deletion results in a less stable protein or the C-terminal part of Mre11 has a specific role in Arabidopsis DNA damage signaling and/or DNA repair. The MRN complex is responsible for the recruitment of ATM to sites of damage during the DNA damage response, which highly depends on the C-terminal part of Nbs1 binding to ATM (Falck *et al.* 2005). Arabidopsis *mre11-2atm-2* mutants are sterile probably because of a defect in meiotic repair (Šamanić *et al.* 2013), similar to the phenotype of *nbs1atm* double mutant plants (Waterworth *et al.* 2007), indicating that the MRN complex and ATM kinase have a redundant function in meiotic recombination and absence of the C-terminal part of Mre11 may impair the interaction between Mre11 (Mre11-Rad50 complex) and Nbs1. However, it has been shown that the Nbs1 binding domain of Mre11 localizes in its N-terminal region in yeast and humans. If this is the case in plants as well the deletion of C-terminus in *mre11-2* probably does not affect the formation of the MRN complex, but it may reduce its interaction activities, as the Mre11 C-terminus was reported to have important protein-protein interaction activities in other organisms.

A *ku80mre11-2* double mutant was generated by crossing the single mutants from different ecotypes. This may have affected the outcome of the results, although this is not likely because the heterozygote ku80+/-mre11-2-/- and ku80+/-mre11-2+/- progeny was used as controls experiments and behaved as expected. We found that the *ku80mre11-2* double mutant was more sensitive to DNA damage than each of the single mutants suggesting that Mre11 may also play a role in b-NHEJ pathway in plant cells and the C-terminal part of Mre11 is important for this function. The subsequent observation of more cell death events in

this double mutant when longer exposed to damage-inducing agents indicates that NHEJ repair pathways are severely disturbed in the concomitant absence of Ku80 and a fully functional Mre11. Beyond its function in activation of the DNA damage response, Mre11 has also been implicated in NHEJ, both in c-NHEJ and also in b-NHEJ that occurs in the absence of c-NHEJ (Zha *et al.* 2009). On the basis of our results we suggest that Mre11 plays an essential role in the b-NHEJ repair pathways in plant cells.

Other evidence supporting a role of Mre11 in b-NHEJ was that the *ku80mre11-2* double mutant was un-transformable by *Agrobacterium*. Using yeast as a model it was shown in our lab that T-DNA integration in yeast (*S.cerevisiae*) is dependent on the non-homologous end joining (NHEJ) pathway of DSB repair, and that proteins such as Ku70, Ku80 and DNA ligase IV are essential for T-DNA integration (van Attikum *et al.* 2001). Inactivation of c-NHEJ in yeast prevented random T-DNA integration, although there is a b-NHEJ pathway in yeast. Putative T-DNA integration by this b-NHEJ pathway may have been beyond the limits of detection. The double strand break repair mechanisms were supposed to be involved in the integration of *Agrobacterium* T-DNA in plants as well. In subsequent studies from other groups and ours on T-DNA integration with Arabidopsis NHEJ mutants T-DNA integrants were invariably obtained, demonstrating that other important factors must be involved in T-DNA integration. The Mre11 might represent such an important protein that plays a critical role in a b-NHEJ pathway involved in T-DNA integration as inactivating both c-NHEJ (Ku80) and b-NHEJ (Mre11) at the same time prevented T-DNA integration in plants. Therefore, Ku80 and Mre11 apparently control two different pathways, each of which is not essential, but together are responsible for all T-DNA integration in plants. A recent study showed that the Arabidopsis Pol θ ortholog Tebichi (Teb) is essential for T-DNA integration (van Kregten et al, 2016). The plant pol θ is able to extend minimally paired 3' ends between the T-DNA left border and the plant genome. However, the mechanism of the attachment of 5' right border of T-DNA to the plant genome remains unclear. Combined with our results, Ku80 and Mre11 probably function together with Pol θ in T-DNA integration in plants. One possibility is that Ku80 or Mre11 are responsible for the capturing T-DNA right border to plant genome.

Acknowledgements

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Table 1. Primers for yeast two-hybrid assays.

Primer	Gene description	Sequence[a]
M11ABDF	At5g54260, Mre11, full length, Fw	5'-TGGAATTCATGTCTAGGGAGGATTTTAGTGA-3'
M11ABDR	At5g54260, Mre11, full length, Rev	5'-CGCTGCAGTTATCTTCTTAGAGCTCCATAGTTC-3'
M11ABDF	At5g54260, Mre11, 1-529 aa, Fw	5'-TGGAATTCATGTCTAGGGAGGATTTTAGTGA-3'
M11NBDR	At5g54260, Mre11, 1-529 aa, Rev	5'-CGCTGCAGTGCTTCCTTTTGTCAAATTCTCTG-3'
M11ABDF	At5g54260, Mre11, 1-499 aa, Fw	5'-TGGAATTCATGTCTAGGGAGGATTTTAGTGA-3'
M114BDR	At5g54260, Mre11, 1-499 aa, Rev	5'-CGCTGCAGCTCTAAGCACTCTCCCACTTTA-3'
M11ABDF	At5g54260, Mre11, 1-510 aa, Fw	5'-TGGAATTCATGTCTAGGGAGGATTTTAGTGA-3'
M11xBDR	At5g54260, Mre11, 1-510 aa, Rev	5'-CGCTGCAGAGTGGGTCGAGTGGACCTATCTTT-3'
M11CBDF	At5g54260, Mre11, 530-720 aa, Fw	5'-TGGAATTCAGTGGCATCGCGAATGCTTCGTTC-3'
M11ABDR	At5g54260, Mre11, 530-720 aa, Rev	5'-CGCTGCAGTTATCTTCTTAGAGCTCCATAGTTC-3'
R50BDF	At2g31970, Rad50, full length, Fw	5'-GGCCATGGAGATGAGTACGGTCGATAAAATG-3'
R50BDR	At2g31970, Rad50, full length, Rev	5'-TTGGATCCTCAATCAAAGATCTCTTGGGCCT-3'
M11AADF	At5g54260, Mre11, full length, Fw	5'-CGGAATTCGAATGTCTAGGGAGGATTTTAGTG-3'
M11AADR	At5g54260, Mre11, full length, Rev	5'-CGCTCGAGTTATCTTCTTAGAGCTCCATA-3'
M11AADF	At5g54260, Mre11, 1-529 aa, Fw	5'-CGGAATTCGAATGTCTAGGGAGGATTTTAGTG-3'
M11NADR	At5g54260, Mre11, 1-529 aa, Rev	5'-CGCTCGAGTGCTTCCTTTTGTCAAATTCTCTG-3'
M11AADF	At5g54260, Mre11, 1-499 aa, Fw	5'-CGGAATTCGAATGTCTAGGGAGGATTTTAGTG-3'
M114ADR	At5g54260, Mre11, 1-499 aa, Rev	5'-CGCTCGAGCTCTAAGCACTCTCCCACTTTA-3'
M11AADF	At5g54260, Mre11, 1-510 aa, Fw	5'-CGGAATTCGAATGTCTAGGGAGGATTTTAGTG-3'
M11xADR	At5g54260, Mre11, 1-510 aa, Rev	5'-CGCTCGAGAGTGGGTCGAGTGGACCTATCTTT-3'
R50AADF	At2g31970, Rad50, full length, Fw	5'-CGGGATCCGAATGAGTACGGTCGATAAAATGTTG-3'
R50AADR	At2g31970, Rad50, full length, Rev	5'-TGCTCGAGTCAATCAAAGATCTCTTGGGCCTCG-3'
N1AADF	At3g02680, Nbs1, full length, Fw	5'-TGGAATTCGAATGGTTTGGGGTCTCTTTCCCG-3'
N1AADR	At3g02680, Nbs1, full length, Rev	5'-CGCTCGAGTCAACTTCCAGAGAGAAACCCGCG-3'

[a]Restriction sites are underlined.

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