



# Kinetic analysis of DNA double-strand break repair pathways in Arabidopsis

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## ABSTRACT

Double-strand breaks in genomic DNA (DSB) are potentially lethal lesions which separate parts of chromosome arms from their centromeres. Repair of DSB by recombination can generate mutations and further chromosomal rearrangements, making the regulation of recombination and the choice of recombination pathways of the highest importance. Although knowledge of recombination mechanisms has considerably advanced, the complex interrelationships and regulation of pathways are far from being fully understood.

We analyse the different pathways of DSB repair acting in G2/M phase nuclei of irradiated plants, through quantitation of the kinetics of appearance and loss of  $\gamma$ -H2AX foci in Arabidopsis mutants. These analyses show the roles for the four major recombination pathways in post-S-phase DSB repair and that non-homologous recombination pathways constitute the major response. The data suggest a hierarchical organisation of DSB repair in these cells: C-NHEJ acts prior to B-NHEJ which can also inhibit MMEJ. Surprisingly the quadruple *ku80 xrcc1 xrcc2 xpf* mutant can repair DSB, although with severely altered kinetics. This repair leads to massive genetic instability with more than 50% of mitoses showing anaphase bridges following irradiation. This study thus clarifies the relationships between the different pathways of DSB repair in the living plant and points to the existence of novel DSB repair processes.

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## 1. Introduction

Double-strand breaks (DSB) in chromosomal DNA can lead to rearrangements and/or loss of chromosomes, with consequences ranging from mutation and malignant transformation to cell death. These highly deleterious lesions are produced accidentally by cellular metabolism and can also be caused by the action of exogenous influences such as chemical mutagens and radiation [1]. By definition, DSB are repaired by recombination through two general classes of mechanism. Homologous recombination (HR) mediates repair using a homologous sequence as template and is a very high fidelity repair process, however, the abundance of non-allelic DNA sequences homologies in many eukaryotic genomes brings the risk of genome rearrangements through ectopic crossing-over. The second general class of recombination mechanism is non-homologous recombination (NHR), which joins DNA ends with little or no dependence on DNA sequence homology (reviews in Refs. [2–5]).

Although knowledge of DNA end-joining DSB repair pathways has greatly advanced in recent years, NHR processes are far from

being completely understood. Different types of joints are found at sites of DSB repair: precise joints and those with minor insertions and/or deletions of the original sequence, or others involving larger deletions and rearrangements. These two types of rejoining result from the involvement of different proteins acting in distinct pathways of NHR. The first class of “high fidelity” events occur through the action of the canonical non-homologous end joining (C-NHEJ) pathway (for reviews in Refs. [4,6]). This pathway begins with break recognition and binding by the KU-heterodimer, broken ends are processed by DNA-PKcs and/or Artemis and ligation carried out by Xrcc4/DNA-Ligase IV associated with Xlf. In the absence of C-NHEJ, precise joints are rare and more complex events often involving recombination of microhomologies are principally found [7–19]. These result from an error-prone pathway(s) of DNA end-joining (EJ), termed alternative-EJ or A-NHEJ. The first proteins described to act in A-NHEJ in *Saccharomyces cerevisiae* were the Mre11-Rad50-Xrs2, implicated in end processing and microhomology annealing, and Rad1-Rad10 (Xpf/Ercc1) in 3' flap removal necessary for filling and sealing of the gap [14,20]. The role of the MRN complex in an alternative NHR pathway using microhomologies has been confirmed in vertebrates, and involvement of Xpf-Ercc1 in a KU-independent end-joining pathway has been also described [21–24]. As this pathway involves recombination between microhomologies flanking the break, it was named microhomology-mediated EJ, or MMEJ. A third NHR pathway involves Parp1, Xrcc1 and DNA Ligase III, known for their roles in DNA single-strand break

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repair [9,25–28]. These proteins act in a KU-independent pathway (called Backup-, or B-NHEJ), preferentially using microhomologies localised at both sides of the break [29] (for review in Refs. [30]). It is not, however, clear whether MMEJ and B-NHEJ are really distinct pathways or manifestations of a complex DSB repair pathway with different redundant, but functionally distinct, activities able to substitute for each other in the different steps leading to joining of a broken DNA molecules (see discussion in Refs. [4,31–33]).

Knowledge of DSB repair pathways in plants remains less developed than in some other biological models and some clear differences have been established, such as the apparent absence in Arabidopsis, and presumably other plants, of known orthologs of some proteins in these pathways (DNA-PKcs, Xlf, Ligase III, Pol $\beta$ ) (discussed in Refs. [34]).

We have recently described the role of Xrcc1 in an alternative NHEJ pathway of DSB repair in Arabidopsis [34]. Our results in Arabidopsis showed that, although severely affected, mutant plants lacking both the KU-dependent and Xrcc1-dependent NHR pathways are able to repair  $\gamma$ -ray induced DSB and here we extend this analysis through study of the roles and interrelations of the major pathways of DSB repair. The data presented here lead us to propose a model of the temporal and hierarchical organisation of the different pathways during DSB repair and show clearly that in the absence of the known DSB pathways, massive chromosome fusions are observed in irradiated cells – pointing to the existence of a fifth pathway.

## 2. Materials and methods

### 2.1. Plants

Conditions for growth of Arabidopsis plants have been previously described [34]. The Arabidopsis *ku80* [35], *xrcc1* [34], *xpf* [36,37] and *xrcc2* [38] mutants and PCR genotyping have been described previously. Crosses between these lines permitted the obtention of double, triple and quadruple mutants.

### 2.2. $\gamma$ -Irradiation

5-day-old seedlings growing on solid medium in petri dishes were  $\gamma$  irradiated using a  $^{137}\text{Cs}$  source (C.I.S. Bio International, Gif-sur-Yvette, France) at an absorbed dose rate of 8.3 Gy/min, and replaced in the growth chamber.

### 2.3. Microscopy

A motorised AxioImager.Z1 (Carl Zeiss AG, Germany) epifluorescence microscope was used to image root tips and mitotic nuclei. The microscope is equipped with an AxioCam Mrm camera and appropriate Zeiss filter sets for the fluorochromes used in this work (Zeiss #49 – DAPI, #47HE – Alexa568). Captured images were further processed and enhanced using Zeiss Axiovision and Adobe Photoshop software.

### 2.4. Root phenotype

Eight days after irradiation, root tips were transferred to slides in a drop of water and covered with a coverslip for bright field microscopy observation.

### 2.5. $\gamma$ H2AX kinetics

Slide preparation, immunostaining and quantification of  $\gamma$ -H2AX foci of mitotic nuclei were carried out as previously described [34,39]. Briefly, at different times following irradiation plantlets were fixed with paraformaldehyde, root tips were

digested and squashed onto slides. Following immunostaining with anti- $\gamma$ -H2AX (and Alexa568 conjugated secondary antibody) and mounting in Vectashield + DAPI, three dimensional image stacks were taken, deconvolved and  $\gamma$ -H2AX foci counted manually. Each point on the graphs is the mean number of  $\gamma$ -H2AX foci per nucleus from counting fifteen nuclei from fifteen to twenty plants. The microscope images presented in Fig. 2 are collapsed Z-stack projections obtained using Extended-focus module (projection method) of the Zeiss Axiovision software.

### 2.6. Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: Ku80 (locus AT1G48050; GenBank NM.103701); Xrcc1 (locus AT1G80420; GenBank NM.106691); Xpf (locus AT5G41150; GenBank NM.123480); and Xrcc2 (locus AT5G64520; GenBank NM.125848).

## 3. Results

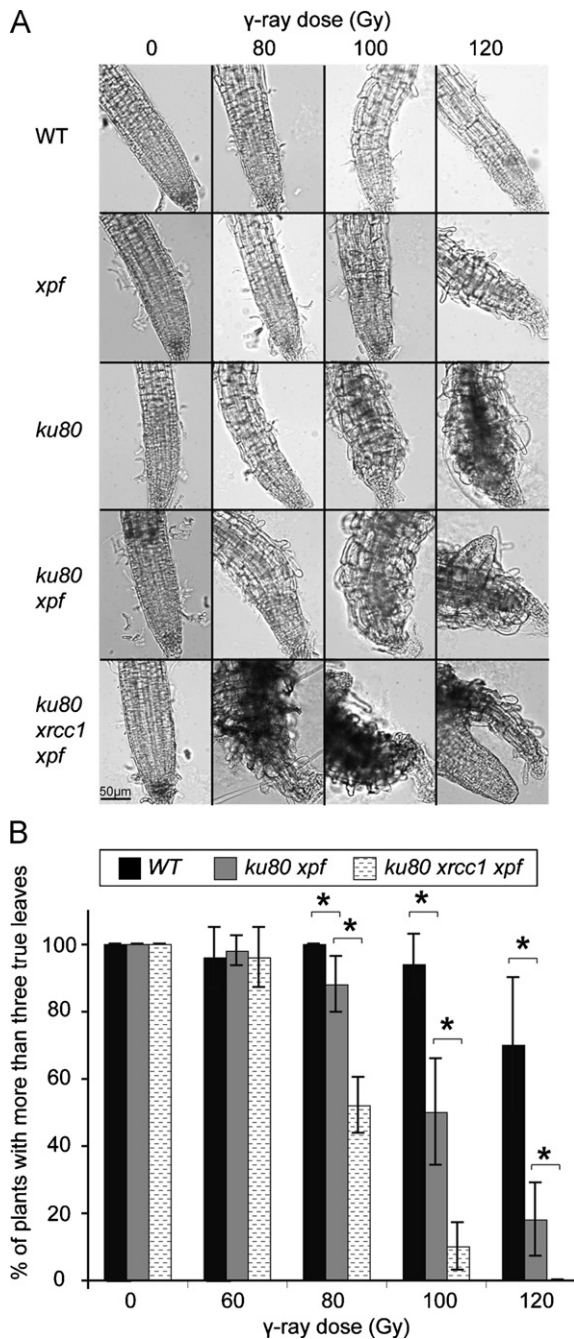
### 3.1. Different sensitivity to ionising radiation for *ku80*, *xrcc1* and *xpf* plants

We have recently identified an Xrcc1-dependent DSB repair pathway in Arabidopsis and shown that *ku80* and *xrcc1* mutants define two non-epistatic DSB pathways in the plant [34]. Notwithstanding the absence of KU-dependent C-NHEJ and Xrcc1-dependent A-NHEJ pathways, G2/M-phase root tip cells of *ku80 xrcc1* Arabidopsis mutants can repair DSB, albeit more slowly. In order to determine which pathway(s) are responsible for this residual Ku80- and Xrcc1-independent repair, we analysed the potential role of the Xpf protein in a third, distinct DSB repair pathway in these cells.

Five-day-old Arabidopsis *ku80*, *xpf*, *ku80 xpf* and *ku80 xrcc1 xpf* mutant seedlings were irradiated with different  $\gamma$ -ray doses from a  $^{137}\text{Cs}$  source (see Section 2 and Ref. [34] for details) and root tips imaged 8 days after irradiation (Fig. 1A). Root tips of *ku80* and *xrcc1* mutant plantlets show severe developmental defects after 100 and 120 Gy, doses which produce little visible effect on WT plantlets (Fig. 1A and Ref. [34]). Much less sensitivity is seen at these  $\gamma$ -ray doses in *xpf* plants, however, *ku80 xpf* plants are clearly more sensitive than either of the single mutants. At doses of 100 and 120 Gy the *ku80 xpf* mutant plants show pronounced cell enlargement and initiation of a new root from tissue immediately above the root tip – indicative of death of the primordial root meristem. The triple, *ku80 xrcc1 xpf*, mutant plants are clearly more sensitive to  $\gamma$ -rays than the *ku80 xpf* double mutant, with the appearance of severe phenotypes from 80 Gy. This  $\gamma$ -ray hypersensitivity is also observed in the production of new leaves, which is clearly more affected in *ku80 xrcc1 xpf* than in *ku80 xpf* plantlets at 80, 100, and 120 Gy (Fig. 1B). The Xpf-dependent pathway is thus non-epistatic to the Ku80-dependent and Xrcc1-dependent pathways of resistance to  $\gamma$ -rays in Arabidopsis.

### 3.2. DNA DSB repair kinetics: determination of three NHEJ pathways

Ionising radiation causes a variety of lesions in DNA in addition to DSB, and in particular produces much more single-strand damage than DSB (approximately 25 SSB for 1 DSB [1]). Thus, although it is clear that the Ku80, Xrcc1 and Xpf proteins are involved in three distinct pathways protecting Arabidopsis from the lethal effects of  $\gamma$ -radiation, these radio-sensitivity data do not show directly that this is due to their roles in three independent pathways of DSB repair. In order to clarify this, we analysed the kinetics of DSB repair



**Fig. 1.** Hypersensitivity of mutant plants to  $\gamma$ -radiation. 5-day-old wild-type and mutant seedlings were  $\gamma$ -irradiated with 0, 60, 80, 100, and 120 Gy. (A) Polarised brightfield images were taken eight days after irradiation, each picture is an example of at least ten roots examined. A 50  $\mu$ m scale bar is included in the lower left image. (B) Proportions of plants with more than three true leaves 8 days after irradiation. Means are of five counts of 10 plants each. Differences significant at  $P < 0.05$  (Student's *t*-test) are marked with an asterisk.

through the quantitation of  $\gamma$ -H2AX foci in dividing root cells of irradiated plants.

Phosphorylation of the variant histone H2AX ( $\gamma$ -H2AX) adjacent to chromosomal DSB is an early step in the response to DSB and their repair. Detection with an appropriate antiserum permits visualisation of  $\gamma$ -H2AX foci in nuclei and the counting of these foci is a powerful tool for quantitative analysis of the breakage and repair of cellular DNA (discussed in Ref. [30]). In previous work, we have used quantitation of the appearance and elimination of  $\gamma$ -H2AX foci after IR exposure to measure the kinetics and efficiency of DSB repair

in wild-type, *ku80*, *xrcc1*, and *xrcc1 ku80* mutant plants [34]. This work both established the kinetics of DSB repair in these cells and permitted the description of an Xrcc1-dependent, KU-independent pathway of DSB repair in Arabidopsis. We present below the application of this approach to specifically determine the role of Xpf and its relationships with Ku80 and Xrcc1 in repair of  $\gamma$ -ray induced DSB in Arabidopsis.

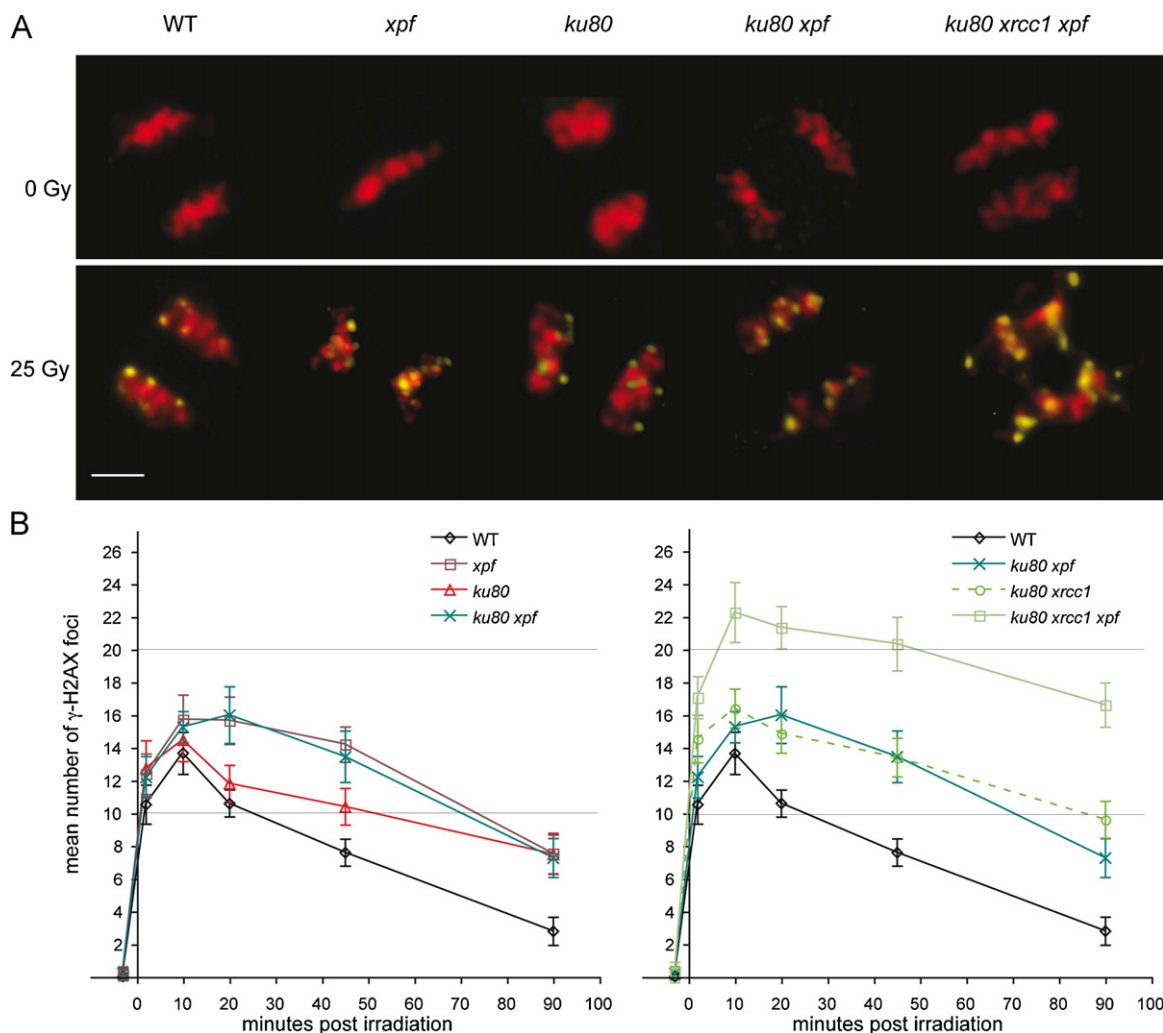
Fig. 2A shows representative images of M-phase mitotic nuclei from root tips of non-irradiated and irradiated Arabidopsis plantlets. Fig. 2B and C shows quantitation of the numbers of these foci across time-courses of repair following irradiation with 25 Gy  $\gamma$ -rays (for clarity the data have been split into two graphs). Results similar to those previously reported were found for wild-type and *ku80* plants (Fig. 2B and Ref. [34]). Absence of the Ku80 protein affects both the very rapid repair (2' time point) and the later phase of repair (after 20'). The repair kinetics are clearly different in the *xpf* mutant, with no detectable removal of foci between 10' and 20' post-irradiation, and the elimination of only one  $\gamma$ -H2AX focus up to the 45' time point. Xpf thus clearly plays an important role in DSB repair in G2/M phase cells of Arabidopsis root tips. Surprisingly, DSB repair in *xpf* plants between 45' and 90' is particularly efficient and comparable to the wild-type in this time window. This is presumably due to the compensatory action of other DSB repair pathways and is in concordance with the weak IR-sensitivity of *xpf* plants (Fig. 1). These data clearly implicate Xpf in DSB repair in Arabidopsis following  $\gamma$ -irradiation and show that the Xpf-dependent pathway is distinct from the KU-dependent pathway.

We were surprised to observe that repair kinetics of *ku80 xpf* plants is very similar to that of the *xpf* single mutant (Fig. 2B). Given the differences in the kinetics of DSB repair in the *ku80* and *xpf* plantlets and the radio-sensitivity data, this is clearly not due to Ku80 and Xpf acting in the same repair pathway (epistasis) and thus a third pathway must compensate for the absence of Ku80 in *xpf ku80* plants.

We have recently demonstrated the role of Xrcc1 in KU-independent DSB repair in Arabidopsis. This KU-independent, Xrcc1-dependent pathway corresponds to "alternative" or "backup" NHEJ and is a logical candidate for the KU-compensatory pathway seen in the absence of Xpf. That this is so is confirmed by analysis of DSB repair in *ku80 xrcc1 xpf*, triple mutant plants, which show a much more severe DSB repair defect than the *ku80 xpf* and *ku80 xrcc1* double mutant lines (Fig. 2C). The *ku80 xrcc1 xpf* nuclei have more  $\gamma$ -H2AX foci at the 2' time-point than *ku80 xpf*, *ku80 xrcc1* and WT plants (17.1 vs. 12.2, 14.5 and 10.5, respectively), and almost 6 times as many foci as WT (16.6 and 2.8 foci, respectively) 90' after irradiation. The triple mutants thus show a considerably more severe deficiency in DSB repair than either *ku80 xpf* or the *ku80 xrcc1* plants (dotted line, from Ref. [34]). These data confirm that Ku80 and Xpf act in separate pathways and that Xrcc1 acts in a pathway that is independent of both the KU-heterodimer and the Xpf/Ercc1 complex. Thus KU-dependent (C-NHEJ), Xpf-dependent (MMEJ + single-strand annealing) and Xrcc1-dependent (B-NHEJ) pathways are simultaneously active in G2/M-phase mitotic cells of Arabidopsis, and very probably in other higher eukaryotes.

### 3.3. Repair efficiency of *xrcc2* plants and the role of homologous recombination in DSB repair

These three NHR pathways are able to repair DSBs very quickly and in their absence we see repair defects mostly at the early, 2 and 10 min post-irradiation time points. At later time points, however, we see clear evidence for DSB repair in mitotic nuclei of *ku80 xrcc1 xpf* plants. Given that our analysis concerns post-S-phase DSB repair (and thus replicated chromosomes), we tested whether this Ku80/Xrcc1/Xpf-independent repair is carried out by homologous recombination.



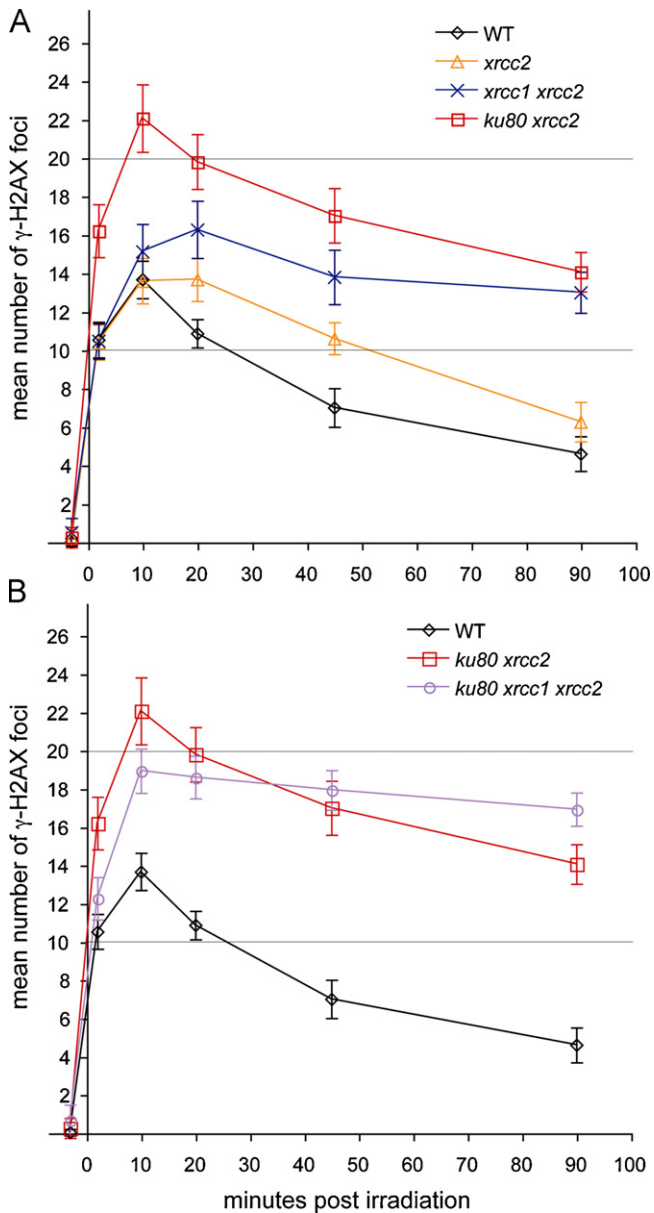
**Fig. 2.** Three NHR pathways in DSB repair. 5-day-old WT, *ku80*, *xpf*, *ku80 xpf* and *ku80 xrc1 xpf* seedlings were  $\gamma$ -irradiated with 25 Gy and root tips stained for DAPI (red) and  $\gamma$ -H2AX (yellow). (A) Representative Z-stack projection images of M-phase nuclei from root tips fixed just prior to (upper), or 20 min after (lower)  $\gamma$ -irradiation. A 2.5  $\mu$ m scale bar is shown at the lower left. (B and C) Numbers of  $\gamma$ -H2AX foci per M-phase nucleus of WT, *ku80*, *xpf*, *ku80 xpf* and *ku80 xrc1 xpf* plants (the *ku80 xrc1* curve (dashed line) comes from Ref. [34]). Each point is the mean of  $\gamma$ -H2AX foci per nucleus from counting fifteen nuclei. Error bars are  $\pm$  standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

To examine the role of homologous recombination in Ku80/Xrcc1/Xpf-independent DSB repair, we tested the involvement of Xrcc2. Xrcc2 is one of the five recombination-mediator, Rad51 paralogue proteins, all of which play important roles in homologous recombination, including Rad51 nucleofilament assembly and stability [40–42]. We have previously identified and characterised mutants for four of these proteins in Arabidopsis. *xrcc2* mutant plants show a strong impairment of somatic homologous recombination (unpublished) and the importance of Rad51D for recombination in Arabidopsis has also been confirmed [38,43–46].

Analysis of DSB repair kinetics in *xrcc2* plants shows a clear defect at intermediate (from 10' to 20') time points following  $\gamma$ -irradiation (Fig. 3A), with fast (up to 10') and slow repair (after 20') not being visibly affected. Xrcc2 (and thus homologous recombination), clearly plays a role in DSB repair in these cells, but its absence can be compensated for by the action of other pathways. Such compensatory action of other pathways is in accordance with the lack of observable  $\gamma$ -ray sensitivity of *xrcc2* plants [38].

In order to confirm that this compensation for the absence of Xrcc2 comes from NHR pathways, we analysed DSB repair kinetics in *ku80 xrcc2* and *xrcc1 xrcc2* double-mutant plants (Fig. 3A). DSB repair in both double mutants is clearly more affected than in the single mutants (Figs. 2 and 3 and Ref. [34]), however, the kinetics

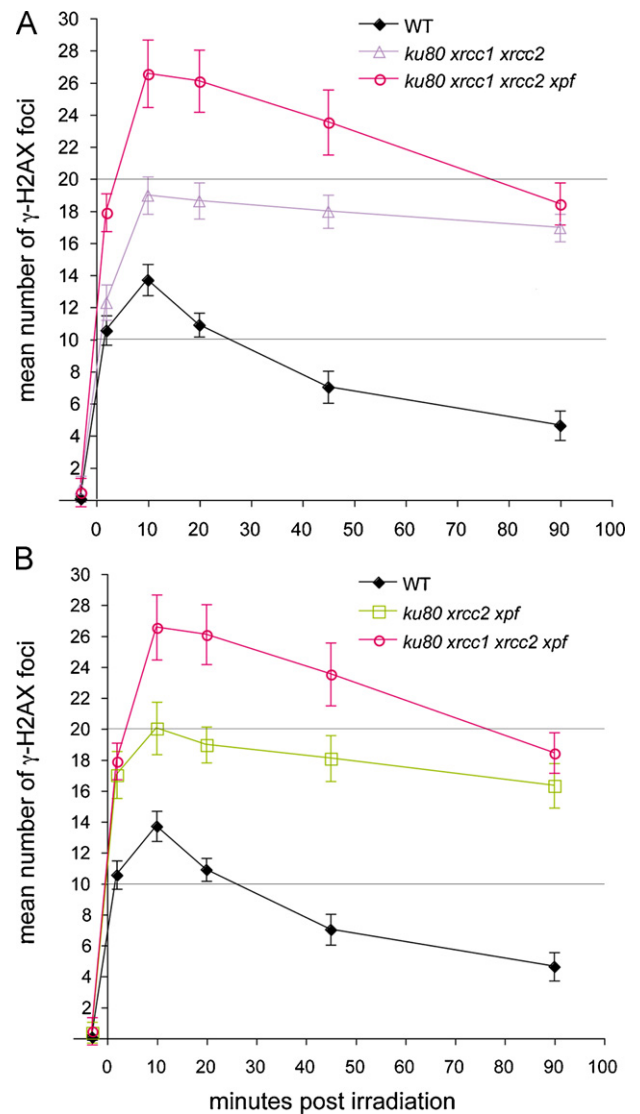
of DSB repair clearly differ between the two double mutant lines. The efficiency of repair in *xrcc1 xrcc2* plants is severely affected between 10' and 90' post irradiation, but fast repair in the first minutes appears similar to WT. In contrast, *ku80 xrcc2* plants show severe repair defects up to 10' after irradiation, with as great an increase in the maximum number of  $\gamma$ -H2AX foci as that observed in triple-mutant *ku80 xrcc1 xpf* plants (Fig. 2C). Following this, however, *ku80 xrcc2* plants repair DSB relatively efficiently with loss of 6  $\gamma$ -H2AX foci (vs. 9 in WT) observed between 10' and 90' post-irradiation. Thus, in G2/M phase cells, homologous recombination is active throughout the time course. In order to confirm these data, we measured repair efficiency in *ku80 xrcc1 xrcc2* plants (Fig. 3B). As expected, these show very severe repair defects throughout the time course with a particularly significant increase in numbers of  $\gamma$ -H2AX foci at early time points (18.9 foci at 10') and practically no detectable repair up to 90' (16.9 foci). Interestingly, the maximum number of foci in the *ku80 xrcc1 xrcc2* plants is less than that observed in the *ku80 xrcc2* double mutant (12.3 and 18.9 foci in *ku80 xrcc1 xrcc2* vs. 16.2 and 22.1 in the *ku80 xrcc2* double mutant at the 2' and 10' time points, respectively). The presence of a functional B-NHEJ pathway in *ku80 xrcc2* plants thus appears to partially prevent the fast repair observed in *ku80 xrcc1 xrcc2* plants during the first minutes following the irradiation.



**Fig. 3.** Role of HR in DSB repair. Mean numbers of  $\gamma$ -H2AX foci per M-phase nucleus of root tip cells 5-day-old seedlings  $\gamma$ -irradiated with 25 Gy. Each point is the mean number of  $\gamma$ -H2AX foci per nucleus from counting fifteen nuclei. Error bars are  $\pm$  standard deviation.

### 3.4. B-NHEJ prevents action of Xpf-dependent DSB repair

Given the demonstration that the Xpf-dependent pathway contributes to DSB repair in the first minutes following irradiation (Fig. 2), the fast repair observed in *ku80 xrc1 xrc2* plants is very probably due to the action of the Xpf-dependent pathway. In order to verify this, we analysed DSB repair kinetics in *ku80 xrc1 xrc2 xpf* quadruple mutant plants. The *ku80 xrc1 xrc2 xpf* mutant plants show a clearly greater number of  $\gamma$ -H2AX foci per nucleus than any of the other mutants tested, with 17.9 and 26.5 foci per nucleus at 2' and 10' post irradiation, respectively (Fig. 4A). These data thus confirm that the fast repair observed in *ku80 xrc1 xrc2* is dependent on Xpf and concord with the observation that the presence of Xrc1 limits the action of the Xpf-dependent pathway (above). Moreover, the ensemble of our data clearly shows the capacity of all three non-homologous recombination pathways, as well as homol-

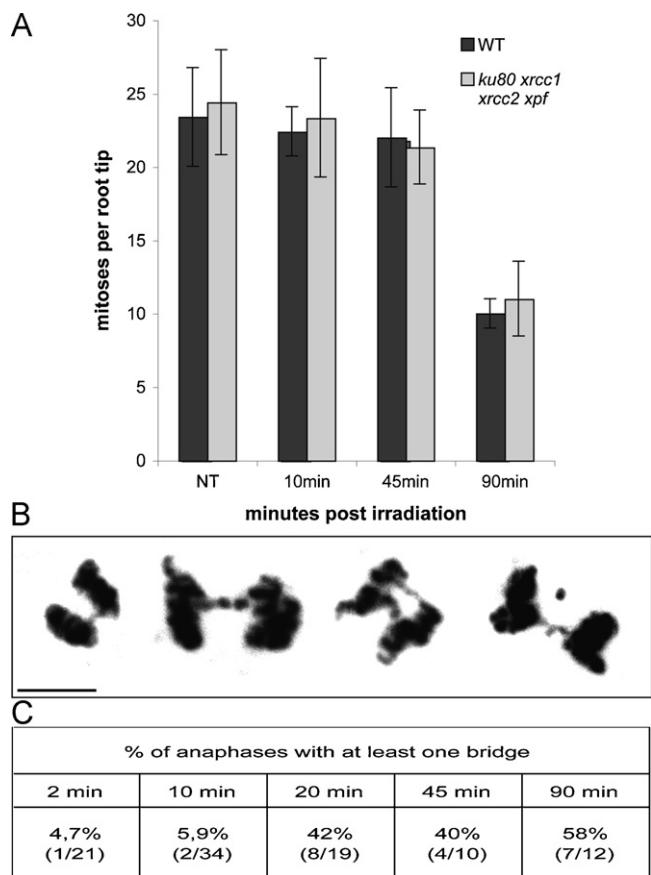


**Fig. 4.** B-NHEJ prevents action of Xpf-dependent DSB repair. Mean numbers of  $\gamma$ -H2AX foci per M-phase nucleus of root tip cells of 5-day-old seedlings  $\gamma$ -irradiated with 25 Gy. Each point is the mean number of  $\gamma$ -H2AX foci per nucleus from counting fifteen nuclei. Error bars are  $\pm$  standard deviation.

ogous recombination, to act in the minutes following irradiation to repair DSB.

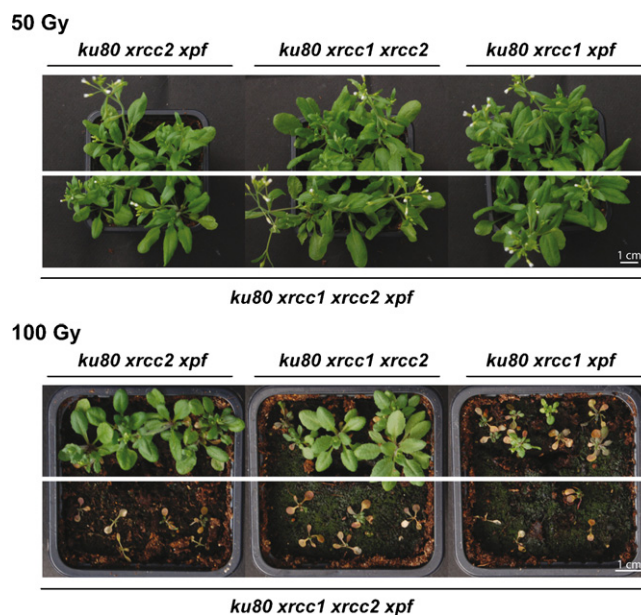
### 3.5. DSB repair in *ku80 xrc1 xpf xrc2* mutants

The analysis of the *ku80 xrc1 xrc2 xpf* quadruple mutant plants unexpectedly shows clear evidence of repair. Notwithstanding the inactivation of all known DSB repair pathways through absence of Ku80 (C-NHEJ), Xrc1 (A-NHEJ), Xpf (MMEJ and SSA) and Xrc2 (HR), time points after 10' show rates of removal of  $\gamma$ -H2AX foci, similar to those of wild-type plants (Fig. 4A). From 10' to 90' post-irradiation, 8.1 foci are lost in the quadruple *ku80 xrc1 xrc2 xpf* mutant and 10.6 lost in WT plants, contrasting markedly with the removal of 2.0 foci during this time period in *ku80 xrc1 xrc2* triple mutant plants. These results point to the action of a novel repair process able to repair DSBs. It is striking to note that this is observed "classical" pathways are not available, as illustrated by the repair kinetics in *xrc1 xrc2* and *ku80 xrc1 xrc2* mutants, which show no repair of DSBs between 45' and 90' after irradiation but have maximal numbers of DSBs/nucleus largely inferior to those of *ku80 xrc1 xrc2 xpf* plants.



**Fig. 5.** DSB repair events in *ku80 xrcc1 xrcc2 xpf* plants. (A) Measurement of mitotic activity following  $\gamma$ -irradiation. 5-day-old seedlings were fixed at different times after 25 Gy  $\gamma$ -rays. Fixed root tips were stained with DAPI and the number of mitoses counted for each root tip. No reduction of mitotic activity is detected up to the 45 min time point. Both wild-type and *ku80 xrcc1 xrcc2 xpf* mutant plants show a significant reduction in the number of mitoses between 45 and 90 min post-irradiation, indicative of activation of the G2/M phase checkpoint. Values are means from 5 root tips for each genotype and time point. NT = non-treated. Error bars are  $\pm$  one standard deviation. (B) Presence of bridges in mitotic anaphases in quadruple mutant after  $\gamma$ -irradiation (DAPI fluorescence in black). A 5  $\mu$ m scale bar is shown at the bottom left. (C) Proportions of mitotic anaphases with at least one visible chromosome bridge at different times following irradiation of *ku80 xrcc1 xrcc2 xpf* plants.

Three control experiments were carried out to confirm that the disappearance of  $\gamma$ -H2AX foci observed in *ku80 xrcc1 xrcc2 xpf* plants is due to recombination. We first verified that this observation is not linked to a particularity of the combination of the *ku80 xrcc2 xpf* mutations. As shown in Figs. 2C and 3, triple *ku80 xrcc1 xpf* and *ku80 xrcc1 xrcc2* mutant plants show strongly reduced repair efficiencies throughout the time-course. That this is also the case for the *ku80 xrcc2 xpf* mutant is shown in Fig. 4B. Thus, none of the three triple mutant combinations (*ku80 xrcc1 xpf*, *ku80 xrcc1 xrcc2* and *ku80 xrcc2 xpf*) show the efficient later repair observed in quadruple mutant, confirming that the action of the novel repair process is only observed when these four mutations are combined. Secondly, we have excluded a possible bias introduced by differential activation of the G2/M phase checkpoint in the WT and the quadruple mutant line. Analysis of mean numbers of M-phase cells per root tip ("mitotic index"), shows similar activation of the G2/M checkpoint in WT and *ku80 xrcc1 xrcc2 xpf* plants at the 90' time point (Fig. 5A). Observation of mitotic anaphases of the quadruple mutant further supports the argument for the removal of  $\gamma$ -H2AX foci in *ku80 xrcc1 xrcc2 xpf* plantlets being due to the action of DSB repair. Incorrect joining of DNA ends through recombination carries the risk of chromatid and chromosome fusions and the dicentric chromosomes that can result are directly observable as chromosome bridges at



**Fig. 6.**  $\gamma$ -Ray sensitivity of triple and quadruple mutants. Photographs of 5-week-old *ku80 xrcc1 xrcc2*, *ku80 xrcc2 xpf* and *ku80 xrcc1 xpf* (upper halves), and *ku80 xrcc1 xrcc2 xpf* (lower halves) plants, irradiated with 50 or 100 Gy 7 days after planting in soil. The quadruple mutant plants are killed by 100 Gy and *ku80 xrcc1 xpf* plantlets are clearly more sensitive than the other two triple mutants. A 1 cm scale bar is shown at the bottom right of each panel.

mitotic anaphase. As seen in Fig. 5, irradiation of *ku80 xrcc1 xrcc2 xpf* plants results in the massive induction of anaphase bridging. A striking, progressive increase in the numbers of anaphase chromosome bridges is observed across the time-course, with more than half (58%) of mitotic anaphase nuclei having at least one visible chromosome bridge at 90' post-irradiation (Fig. 5B and C). This value is even higher than that observed in wild-type plants under these conditions: 37.8% (14/37) mitoses with at least one visible bridge at 90 min post-irradiation.

That this massive induction of dicentric chromosomes corresponds to aberrant DSB repair is confirmed by the  $\gamma$ -radiation sensitivities of these lines (Fig. 6). Growth of the triple *ku80 xrcc1 xpf* and quadruple mutant *ku80 xrcc1 xrcc2 xpf* plants is severely affected by 100 Gy  $\gamma$ -rays. The quadruple mutant plantlets are most sensitive (killed at 100 Gy) and *ku80 xrcc1 xpf* clearly more sensitive than the other two triple (*ku80 xrcc1 xrcc2* and *ku80 xrcc2 xpf*) mutants. As both the *ku80 xrcc1 xpf* and *ku80 xrcc1 xrcc2 xpf* mutants lack functional NHR pathways, these data thus also confirm the predominant role of NHR repair pathways in the response to IR induced damage in Arabidopsis.

#### 4. Discussion

We have recently described the role of Xrcc1 in an alternative NHEJ pathway of DSB repair in Arabidopsis [34]. This is presumably equivalent to the Xrcc1-dependent "backup-NHEJ" (B-NHEJ) pathway, described in mammalian cells. In that work we noted that our radiosensitivity and gamma-H2AX data appeared to contrast with an earlier comet-assay analysis describing important roles for Rad21 and Smc6 (Mim) Arabidopsis orthologs [47,48]. Direct comparison of the different studies is difficult, but it is likely that the differing cell-types (the comet assay measures nuclei from a mixed population of cell types and cell-cycle phases, with presumably a majority of non-cycling cells) and differences between the bleomycin treatment used (1 h, unknown number of DSBs) vs. gamma-irradiation (3 min, 15 DSBs per 4C nucleus), may have affected quantification of the early time points and so masked the

early role of Ku80 (and Lig4) (discussed in Ref. [47]). Our results in Arabidopsis showed that the Xrcc1-dependent pathway contributes as efficiently as KU-dependent C-NHEJ to DSB repair and is thus not simply a “backup” pathway in Arabidopsis. Although severely affected, mutant plants lacking both the KU-dependent and Xrcc1-dependent NHR pathways are, however, able to repair DSB and in this work we set out to identify the other pathways involved and the relationships between these.

#### 4.1. Involvement of Xpf in several recombination pathways: confirmation of MMEJ in plants

The Xpf-Ercc1 structure-specific endonuclease is best known for its role in nucleotide excision repair, where it cleaves 3' of a double-strand to single-strand transition in DNA in the repair of distorting lesions in DNA, such as UV-induced pyrimidine dimers. Xpf/Ercc1 is also involved in recombination and the repair of DSB through single-strand annealing (SSA) of flanking direct repeats, as initially demonstrated in yeast [49] and since confirmed in other eukaryotes (reviewed in Ref. [3]), including Arabidopsis [36,50]. Xpf plays an essential role in micro-homology mediated end-joining (MMEJ) in yeast and mammals [14,21], although this has not yet been tested in Arabidopsis. Evidence for a functional MMEJ pathway in Arabidopsis comes from the observation of reduced usage of microhomologies in chromosomal end-to-end fusions in *ku70 tert mre11*, compared to *ku70 tert* plants [51].

The kinetics of DSB repair in *xpf* plants presented here clearly indicates an important role of the Xpf protein in DSB repair following IR exposure or Arabidopsis plantlets. This is underlined by the greater severity of DSB repair defects observed in *xpf* plantlets than in *ku80* mutants. The data presented here suggest that the Xpf/Ercc1 complex also plays a role in MMEJ in Arabidopsis and show that this pathway acts rapidly in repair of DSB in the first minutes following gamma irradiation in Arabidopsis.

#### 4.2. Three distinct non-homologous DSB repair pathways

Although a number of reports concerning KU-independent, A-NHEJ repair processes and proteins in higher eukaryotes have been published in recent years, a certain confusion remains concerning the specific roles and the relations between the different non-homologous recombination pathways. In order to address this we have analysed *ku80* (C-NHEJ), *xrcc1* (SSBR-related A-NHEJ), and *xpf* (MMEJ) mutant plants and different multiple mutants combining these mutant alleles.

Analysis of sensitivity to ionising radiation of *ku80*, *xrcc1*, *xpf* and triple, *ku80 xrcc1 xpf*, mutant plants shows the involvement of Ku80, Xrcc1, and Xpf in at least three distinct repair processes in response to  $\gamma$ -irradiation. The sensitivity of *ku80* and *xpf* plants to DNA breakage has been previously described [35,43,52,53]. We confirm the  $\gamma$ -ray hyper-sensitivity of *xpf* and *ku80* plants and show that the double *ku80 xpf* mutants are more sensitive than either single mutant. Ku80 and Xpf are thus involved in two distinct processes in response to gamma irradiation. These data concord with observations in mammals, in which *ku86 ercc1* fibroblasts are more sensitive than either single mutant to IR [21]. That the Xpf-dependent pathway is distinct from both the KU-dependent and Xrcc1-dependent pathways, is shown by the greater  $\gamma$ -ray sensitivity in the *ku80 xrcc1 xpf* triple mutant than that of *ku80 xpf* mutants. *ku80 xrcc1 xpf* plants also show more severe repair defects in DSB repair kinetics relative to both *ku80 xpf* and *ku80 xrcc1*, confirming that the higher IR sensitivity of *ku80 xrcc1 xpf* plantlets is due to defects in DSB repair (this study, [34]). The Ku80, Xpf and Xrcc1 proteins are thus involved in at least three distinct repair processes in response to IR and three NHR pathways thus contribute significantly to DSB repair in Arabidopsis and very likely in other higher

eukaryotes (KU-dependent C-NHEJ, Xrcc1-dependent B-NHEJ and Xpf-dependent MMEJ).

DSB repair kinetics and radiosensitivities of the mutant plants show that these three NHR pathways act rapidly in the first minutes following irradiation and constitute the major response to DSB formation induced by  $\gamma$ -radiation. We note that our analyses concern post-replicative phases of the cell cycle and thus show a predominant role of NHR in DSB repair, notwithstanding the availability of sister chromatids. This observation in Arabidopsis is in agreement with studies in mammals showing that NHR processes are active throughout the cell cycle, and that HR seems to play a relatively minor role in post-S-phase DSB repair [10,54–57].

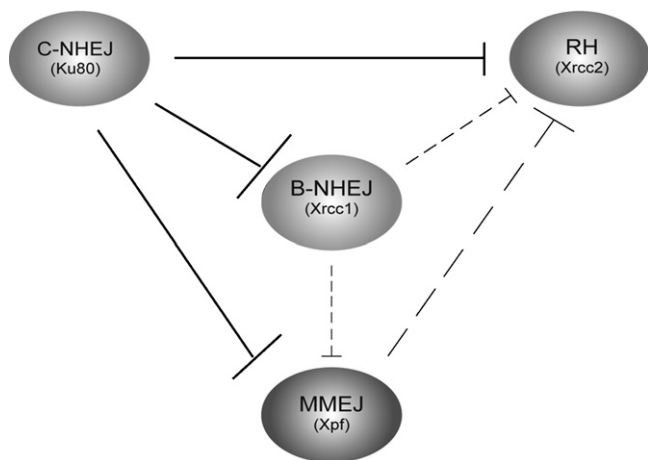
#### 4.3. Role of Xrcc2 and homologous recombination during DSB repair

In order to clarify the respective contributions of NHR and HR pathways in post-S-phase DSB repair, we analysed the potential role of Xrcc2. DSB repair kinetics of *xrcc2* mutant plants show a role of the Xrcc2 protein in repair of  $\gamma$ -ray induced DSB in Arabidopsis. Xrcc2 (and thus HR), however, plays a relatively minor role in response to repair of DSB induced by gamma radiation, at least at the doses used here. This concurs with the lack of  $\gamma$ -ray hypersensitivity we reported in *xrcc2* mutants [38]. Homologous recombination is a relatively slow process with, for example, mating type switching in yeast taking an hour to complete [58,59] and IR-induced Rad51 foci being observable after 30 or 60 min in mammalian and yeast cells [60,61]. A comparison of NHEJ and HR in human cells has shown that reconstruction of a chromosomal GFP marker gene takes 30 min by NHEJ and 7 h by HR [56]. We show in this work, however, that an HR-dependent process is able to cause loss of gamma-H2AX foci in a few minutes in the absence of KU. This observation raises the possibility that inhibition of this HR process by KU could explain the “slowness” of HR repair observed in other organisms and could well contribute to the effect of absence of C-NHEJ increasing HR in mammals (reviewed in Ref. [62]). It is important to note here that the loss of  $\gamma$ -H2AX foci does not necessarily directly measure the completion of repair and in the case of MAT switching in yeast, loss of  $\gamma$ -H2AX correlates with the beginning of DNA synthesis from the 3' invading end on the donor template [63]. Notwithstanding, this very rapid action of HR in DSB repair remains surprising and more work is needed to examine the mechanistic basis for this observation.

#### 4.4. Hierarchy of action of the DSB repair pathways

DSB repair kinetics of *ku80 xrcc2*, *xrcc1 xrcc2* and *ku80 xrcc1 xrcc2* cast light on the relationships between the C-NHEJ, B-NHEJ and MMEJ pathways during DSB repair (Fig. 3A and B). C-NHEJ acts very rapidly in the first minutes following irradiation and prevents action of B-NHEJ during this kinetic window. The involvement of B-NHEJ is visible in later repair events, when the KU-dependent pathway is less effective. In a similar manner, the Xrcc1-dependent pathway limits the action of the Xpf-dependent pathway(s), MMEJ (and SSA?), as shown by the fact that B-NHEJ prevents the action of MMEJ in the first minutes post-irradiation in *ku80 xrcc2*, compared to *ku80 xrcc1 xrcc2* plants (Fig. 3A and B).

These data lead us to propose a model of the temporal organisation of the different pathways during DSB repair (Fig. 7). C-NHEJ would come to DSBs first. The presence of the proteins of this pathway, and in particular KU, would prevent access and action of other DSB repair processes, such as Xrcc1-dependent B-NHEJ. Saturation (or absence) of the KU-dependent pathway would permit the action of the other pathways. Similarly, B-NHEJ limits the contribution of MMEJ (and SSA?) during DSB repair and finally, HR would act on DSBs which were not processed by the NHR pathways. This



**Fig. 7.** Model of hierarchical organisation of pathways during DSB repair. Hierarchical organisation of the different pathways during post-S-phase DSB repair in Arabidopsis. The C-NHEJ pathway, and in particular KU, restricts access and action of other DSB repair processes. In case of saturation (or absence) of the KU-dependent pathway, A-NHEJ acts and limits the access of MMEJ (and SSA?) to lesions. HR would act on DSBs not dealt with by the three NHR pathways.

model is in agreement with published data showing the importance of KU heterodimer in the choice of the pathway for DSB repair [7,8,10,12,13,15,19,22,23,28,64–69]. In addition, as in the data presented here, recent reports show the inhibitory effect of the KU heterodimer on other DSB repair processes, such as A-NHEJ and HR [15,31,33,64,66,70].

The approach taken in this work thus both permits, and underlines the importance of, establishment of the functional hierarchy of the different pathways and activities *in vivo* in response to a complex challenge to genome integrity. It is of course possible that the absence of a specific repair protein or pathway affects not only the processing of directly induced DSB, but also affects the generation of DSB as secondary lesions. Such effects do not change our conclusions as absence of a specific repair protein or pathway leading to the generation of DSB as secondary lesions, is clearly evidence for their role(s) in the response to DNA breakage. This argument is applicable to all *in vivo* indirect studies of breakage and their repair, and is a necessary consequence of the use of treatments producing multiple types of lesions and their study in mutants. The alternative to induce a specific lesion by endonuclease cleavage (for example), is a powerful tool for a specific type of lesion (at a given genomic locus) but severely limits conclusions concerning “real world” functioning of multiple pathways faced with multiple types of lesions in multiple genomic contexts.

#### 4.5. A novel DSB repair process the absence of the four major DSB repair pathways

Analysis of DSB repair kinetics of *ku80 xrcc1 xrcc2 xpf* plants, inactivated for all known DSB repair processes (C-NHEJ, B-NHEJ, MMEJ, SSA and HR), shows unexpectedly that the quadruple mutant plants are able to repair DSBs and between 10 and 90 min after irradiation show a repair efficiency similar to the wild type. This repair is not observed in the three triple mutant lines and thus presumably comes from a “novel” DSB repair process, different from the C-NHEJ, B-NHEJ, MMEJ, SSA or HR pathways. Very high levels of chromosome fusions and anaphase bridging induced by  $\gamma$ -rays in the quadruple mutant show that action of this pathway confers severe genomic instability. The nature of this pathway and whether or not it exists in other eucaryotes is not known at this time, although possibilities are suggested by the KU-independent association of DNA Ligase IV with chromatin [71] and recent reports of

a replication template-exchange chromosome fusion mechanism [72–74].

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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