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Different functional roles of RTR complex factors in DNA repair and meiosis in Arabidopsis and tomato

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SUMMARY

The RTR (RecQ/Top3/Rmi1) complex has been elucidated as essential for ensuring genome stability in eukaryotes. Fundamental for the dissolution of Holliday junction (HJ)-like recombination intermediates, the factors have been shown to play further, partly distinct roles in DNA repair and homologous recombination. Across all kingdoms, disruption of this complex results in characteristic phenotypes including hyper-recombination and sensitivity to genotoxins. The type IA topoisomerase TOP3α has been shown as essential for viability in various animals. In contrast, in the model plant species Arabidopsis, the *top3α* mutant is viable. *rmi1* mutants are deficient in the repair of DNA damage. Moreover, as opposed to other eukaryotes, TOP3α and RMI1 were found to be indispensable for proper meiotic progression, with mutants showing severe meiotic defects and sterility. We now established mutants of both *TOP3α* and *RMI1* in tomato using CRISPR/Cas technology. Surprisingly, we found phenotypes that differed dramatically from those of Arabidopsis: the *top3α* mutants proved to be embryo-lethal, implying an essential role of the topoisomerase in tomato. In contrast, no defect in somatic DNA repair or meiosis was detectable for *rmi1* mutants in tomato. This points to a differentiation of function of RTR complex partners between plant species. Our results indicate that there are relevant differences in the roles of basic factors involved in DNA repair and meiosis within dicotyledons, and thus should be taken as a note of caution when generalizing knowledge regarding basic biological processes obtained in the model plant Arabidopsis for the entire plant kingdom.

Keywords: *Arabidopsis thaliana*, *Solanum lycopersicum*, topoisomerase, helicase, dissolution.

INTRODUCTION

Ensuring genome stability during DNA replication and chromosome segregation is a fundamental necessity for all eukaryotes to prevent adverse defects and potential cell death. Therefore, essential repair mechanisms have evolved that are highly conserved throughout all kingdoms. Homologous recombination (HR) is a mechanism employed to repair double-strand breaks, in addition to replicative DNA damage. Multiple pathways of HR exist in somatic cells, as well as in meiosis. As an outcome of HR, either a crossover (CO) or non-crossover (NCO) may arise. As repair intermediates, joint molecules such as dHJs are often formed and their subsequent entanglement is essential for correct chromosome separation and thus cell survival. The dissolution pathway, mediated by the RTR complex comprised at least of a RecQ helicase, a type IA topoisomerase and the structural protein Rmi1 (RecQ-mediated genome instability), greatly contributes to the resolution of repair intermediates, resulting exclusively in NCO product outcomes (Knoll *et al*., 2014; Wu and Hickson, 2003).

During dissolution, the activity of the RecQ helicase mediates migration of the junctions of dHJs, forming a hemicatenane structure (reviewed in Dorn and Puchta, 2019). Stabilized by Rmi1, the topoisomerase is recruited and cleaves the joints of this structure, via its action as a decatenase, unlinking the DNA molecules to generate a NCO (Yang *et al*., 2010).

Owing to the fundamental role of the RTR complex in maintaining genome stability, it is not surprising that it is highly conserved throughout eukaryotes, with homologues of the complex partners prevalent in yeast, animals and plants (Wu and Hickson, 2003).

The RecQ helicase Sgs1, topoisomerase 3 (Top3) and Rmi1 are the interacting RTR complex partners in *Saccharomyces cerevisiae*. Yeast cells deficient in Sgs1 were shown to exhibit hypersensitivity against genotoxic agents and a hyper-recombination phenotype (Onoda *et al*., 2000). Genetic mutations disrupting the human *Sgs1* homologue *BLM*, culminates in growth retardation and an increased susceptibility to cancer. This hereditary disease, known as

Bloom's syndrome, is the result of enhanced sister chromatid exchanges due to a highly elevated HR rate (Chaganti *et al*., 1974; Ellis *et al*., 1995; German, 1993). These elevated recombination frequencies and increased genotoxin sensitivities were ultimately shown to be the characteristic phenotypes for mutants lacking any of the RTR complex partners in different species.

RECQ4A was demonstrated to act as the functional homologue of ScSgs1 and HsBLM in the plant model organism, *Arabidopsis thaliana*. This helicase was further shown to be instrumental in somatic DNA repair in plants, with its loss resulting in the characteristic phenotypes of RTR complex partners of elevated HR and hypersensitivity to genotoxins (Bagherieh-Najjar *et al*., 2005; Hartung *et al*., 2000; Knoll and Puchta, 2011; Mannuss *et al*., 2010; Schröpfer *et al*., 2014). Arabidopsis also harbours an additional paralogue, RECQ4B; however no apparent DNA repair defects have been elucidated for mutants of *RECQ4B*, despite the highly conserved domain structure and sequence similarity to RECQ4A (Hartung *et al*., 2007). Nevertheless, double mutants of both RECQ4 paralogues in Arabidopsis have been shown to depict a six-fold increase in COs, highlighting the extent of their meiotic roles in suppressing CO frequencies (Séguéla-Arnaud *et al*., 2015). This knowledge could also be transferred to crop plants, as an increase in CO frequency was demonstrated in *recq4* mutants of rice, tomato and pea (Maagd *et al*., 2020; Mieulet *et al*., 2018).

Arabidopsis mutants lacking *RMI1* also demonstrate sensitivity to DNA damaging agents, and increased HR similar to that observed for plants deficient in *RECQ4A* (Bonnet *et al*., 2013; Hartung *et al*., 2008). Therefore, although having no catalytic function, RMI1 was also demonstrated as an important factor for DNA repair in plants. Despite not being present in yeast, a second RMI protein, RMI2 is also an integral factor of the Arabidopsis and mammalian RTR complex (Röhrig *et al*., 2016; Singh *et al*., 2008; Xu *et al*., 2008). Characteristic of mutants of all RTR complex partners, *rmi2* mutants in Arabidopsis exhibit hyper-recombination and genotoxin sensitivity (Röhrig *et al*., 2016).

Intriguingly, mutant phenotypes for *Top3* homologues vary strongly in different organisms. In baker's yeast, *top3* mutants exhibit slower growth than wild-type (WT) and sporulation defects, although still viable as opposed to fission yeast mutants (Gangloff *et al*., 1994) Multicellular eukaryotes harbour two Top3 homologues, TOP3α and TOP3β. However, only TOP3α acts as a true ScTop3 homologue, whereas a role as an RNA topoisomerase was postulated for TOP3β (Xu *et al*., 2013). TOP3α was demonstrated as essential in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster* with severe mutant phenotypes ranging between complete embryo lethality and premature death during early developmental stages (Kim *et al*., 2000; Li and Wang, 1998; Plank *et al*., 2005).

Interestingly, the *top3α* null mutant phenotype in Arabidopsis was unclear for over 10 years (Hartung *et al*., 2008; Hartung *et al*., 2007). Differing phenotypes from two T-DNA mutant lines caused confusion that was only recently resolved by the application of CRISPR/Cas9-mediated mutagenesis. Knockout of *TOP3α* in Arabidopsis revealed a surprising viable mutant phenotype (Dorn *et al*., 2018). Thus, TOP3α plays a significant role in plant DNA repair with mutants displaying a number of somatic defects including dwarfism, fasciated organs and increased cell death within the root meristem due to replication-associated DNA damages, on top of the classical RTR mutant phenotypes (Dorn *et al*., 2018; Hartung *et al*., 2008).

Astonishingly, an unforeseen outcome following analysis in plants revealed that both TOP3α and RMI1 are essential for meiosis in Arabidopsis, with plants lacking one or the other protein being rendered sterile (Chelysheva *et al*., 2008; Dorn *et al*., 2018; Hartung *et al*., 2008). Dual roles for both TOP3α and RMI1 in somatic and meiotic DNA repair were surprising as this has not been observed for all eukaryotic organisms, with studies only showing related defects occurring in *S. cerevisiae* and *C. elegans* (Gangloff *et al*., 1999; Goodwin *et al*., 1999; Wicky *et al*., 2004). Catastrophic meiotic defects were observed for Arabidopsis mutant lines of both genes, with extensive chromosome fragmentation and chromatin bridges leading to meiotic arrest after meiosis I (Chelysheva *et al*., 2008; Hartung *et al*., 2008). The extent of this meiotic damage is speculated to be because of the accumulation of unresolved recombination intermediates. The subcomplex that RMI1 and TOP3α forms is likely required to dissolve these as part of its essential role in ensuring proper chromosome entanglement and segregation (Chelysheva *et al*., 2008; Dorn *et al*., 2018; Hartung *et al*., 2008). Moreover, this subcomplex was demonstrated to suppress meiotic CO formation in Arabidopsis (Séguéla-Arnaud *et al*., 2017).

As integral factors of both DNA repair and meiosis, shown by studies carried out in Arabidopsis, TOP3α and RMI1 are considered of great interest for further translational approaches to agronomically important crop species. To determine whether the somatic and meiotic functions of the RTR complex partners, TOP3α and RMI1, observed for Arabidopsis are conserved in other plant species, we generated Cas9-mediated mutants of both factors in the economically important crop species, *Solanum lycopersicum*. Utilizing a multidisciplinary approach, analysis of these mutant lines demonstrated unexpected differences between the potential roles of these factors, compared with those known in Arabidopsis. This highlights that differences can be found even between dicotyledonous species of plants and emphasizes the increased need of functional gene analysis in crop plants.

RESULTS

CAS9-mediated mutagenesis of TOP3α and RMI1 tomato homologues

Owing to the dual roles within meiosis and DNA repair for both TOP3α and RMI1 that were shown in Arabidopsis (Chelysheva *et al.*, 2008; Dorn *et al.*, 2018; Hartung *et al.*, 2008), it was interesting to see whether this was also the case for the agronomically important crop plant tomato (*S. lycopersicum* L.). As a result, Cas9-mediated mutagenesis of these genes was carried out to generate mutant lines, using the tomato cultivar Micro-Tom as a model system (Campos *et al.*, 2010).

First, homologous genes of both *AtTOP3α* (At5g63920) and *AtRMI1* (AT5G63540) within the tomato genome were identified. Homology searches were conducted using Arabidopsis sequence data. For *RMI1*, the gene Solyc12g005900.2 on chromosome 12 was identified, which is 2389 bp in length and comprised of eight exons, with 49.26% sequence identity on DNA level and 46.13% on protein level to *AtRMI1* (Figure 1b). In terms of conserved functional domains of the protein, Solyc12g005900.2.1 (herein referred as SlRMI1), contains both the DUF1767 domain and the OB-fold domain 1 (Figure 1a), both shown to be essential for the DNA repair and meiotic roles of RMI1 in Arabidopsis (Bonnet *et al.*, 2013). In addition, Solyc11g066690.2 on chromosome 11 was identified as homolog of At*RMI2* (At1g08390), which confirms the presence of both RMI proteins in tomato.

For TOP3α, the homologous protein Solyc05g014720.3.1 (SlTOP3α) was identified following homology searches with the Arabidopsis orthologue. The gene encoding this protein is located on chromosome 5 of the tomato genome, 3564 bp in length with a sequence identity of 68.43% to At*TOP3α* and made up of 24 exons (Figure 2b). The protein sequence of 915 amino acids has 70.04% identity to that of AtTOP3α, and has the conserved domains known to be essential for the function of the topoisomerase in plants, including the TOPRIM domain and central domain with the catalytic tyrosine residue (Dorn *et al.*, 2018) (Figure 2a).

For mutagenesis of both *SlRMI1* and *SlTOP3α*, the CRISPR/Cas9 constructs discussed in Fauser et al. (2014) were used. The resistance cassette was changed within the pDe-SpCas9 destination vector from a phosphinothricin cassette to a kanamycin one for transgenic selection. Target sequences within the first exons for both genes were identified and the spacer sequences (SlRMI1 Protospacer 5′-TTCGGATTGTGGTATTGGTC-3′, SlTOP3α Protospacer 5′-GATGCTGTTCACATCTGTCA-3′) were cloned into the CRISPR/Cas9 expression constructs. Following Agrobacterium-mediated transformation and subsequent tissue culture techniques, the seeds of the regenerated transgenic plants (T0) were harvested to obtain the T1 generation. DNA from the seedlings of the T1 generation, from several independently transformed lines, was extracted and used to screen for plants harbouring induced mutations, using high-resolution melting (HRM) analysis and Sanger sequencing. From this, T1 plants with mutations and a single locus copy of the T-DNA were further propagated to obtain the T2 generation. Further generations were ensured to be transgene-free and propagated to obtain a higher number of seeds for analysis.

Null mutants of *RMI1* in tomato are fertile

Two different, independent mutant lines, each with heterozygous mutations within *SlRMI1*, were identified in the T1 generation, named *Slrmi1-1*, with a 1-bp insertion and *Slrmi1-2* with a 4-bp deletion in exon 1, respectively. The mutations for both of these lines lead to a frameshift within the open reading frame of the *RMI1* gene, and therefore gave rise to premature stop codons. The mutations were confirmed on mRNA level by Sanger sequencing of the cDNA (Figure S1). Within the T2 generation, homozygous mutant plants for both lines were identified and used for subsequent analysis. Plants of both mutant lines were indistinguishable from WT throughout growth from seedling to mature plant with flowers and ripe fruits (Figure S2). Surprisingly, the plants were not sterile, as anticipated, as seeds were obtained from fruits for both lines. This was not expected due to the known sterility of the *Atrmi1* mutants, as previously described (Chelysheva *et al.*, 2008; Hartung *et al.*, 2008).

Although not sterile, it was speculated that the homozygous *Slrmi1* mutant lines would show some level of reduced fertility, due to the meiotic catastrophe and sterility observed for Arabidopsis *rmi1* mutants (Bonnet *et al.*, 2013; Chelysheva *et al.*, 2008; Hartung *et al.*, 2008). Therefore, analyses were conducted to determine fertility of the mutants, in relation to WT plants. First, the average number of seeds per fruit were analysed for both lines, compared with that of WT. In doing this, it was shown that there is no significant difference between both mutant lines and WT, or the two mutant lines themselves (Figure 3a). Furthermore, to ascertain pollen viability in *Slrmi1*, fluorescein diacetate (FDA) staining was performed. With this assay, fluorescing pollen are deemed viable whereas non-fluorescent pollen are considered metabolically inactive and non-viable. The fluorescent and non-fluorescent pollen were quantified to determine the percentage of viable pollen grains. Owing to no significant difference between the two mutant lines and WT (Figure 3b), it appears that a lack of RMI1 does not result in reduced pollen spore viability. Meiotic progression of Arabidopsis *rmi1* mutants is completely abolished due to severe chromosome fragmentation and improper chromosome segregation, with anaphase bridges and arrest before meiosis II (Chelysheva *et al.*, 2008; Hartung *et al.*, 2008). The essential

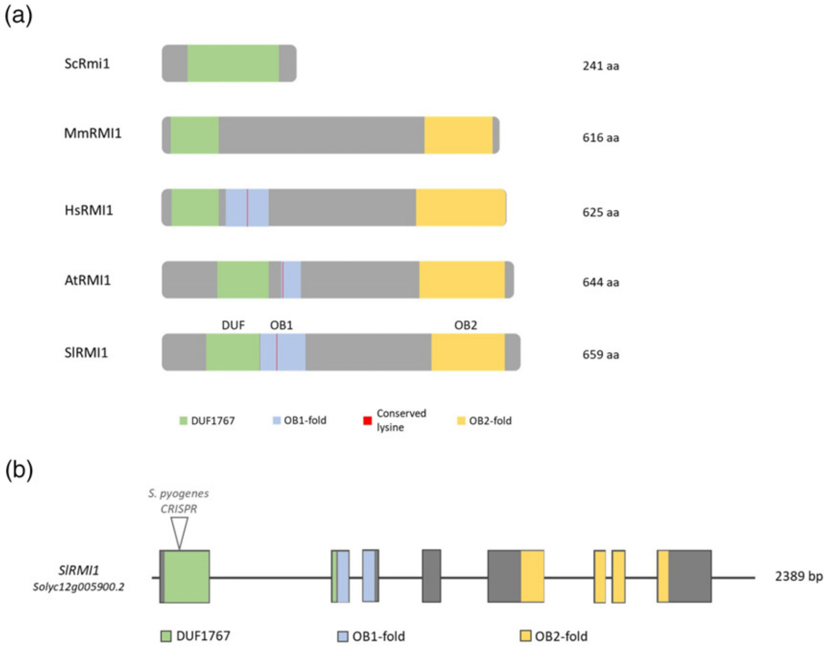

Figure 1. Gene structure and protein domain comparison of the tomato RMI1 orthologue with homologues from other eukaryotes.
(a) Schematic diagram illustrating the conserved protein domains in RMI1 orthologues from *Saccharomyces cerevisiae* (Sc, baker's yeast), *Mus musculus* (Mm, mouse), *Homo sapiens* (Hs, human), *Arabidopsis thaliana* (At, Arabidopsis) and the identified orthologue in *Solanum lycopersicum* (Sl, tomato). The tomato RMI1 protein is 659 amino acids (aa) in length and contains the three domains known to be important for the function of RMI1 in *A. thaliana* (Bonnet *et al.*, 2013) (DUF: aa 83–177; OB1: aa 175–260; OB2: aa 496–638). Red line indicates the conserved lysine that is a known essential aa for interaction of RMI1 with TOP3α (K220 in *S. lycopersicum*).
(b) *SlRMI1* gene structure indicating exons (boxes), domain structure within these and untranslated regions including introns as a line. *RMI1* gene in tomato is comprised of eight exons and is 2389 bp in length. Site within exon 1 is indicated by the arrow, where Cas9 was targeted to via the spacer sequence for induced mutagenesis.

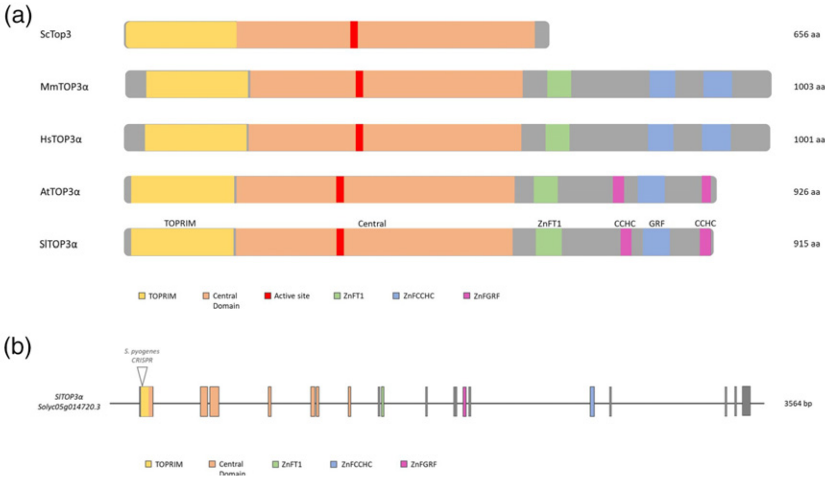

Figure 2. Gene structure and protein domain comparison of the tomato TOP3α orthologue with homologues from other eukaryotes.
(a) Schematic diagram illustrating the conserved protein domains in TOP3α orthologues from *Saccharomyces cerevisiae* (Sc, baker's yeast), *Mus musculus* (Mm, mouse), *Homo sapiens* (Hs, human), *Arabidopsis thaliana* (At, Arabidopsis) and the identified orthologue in *Solanum lycopersicum* (Sl, tomato). Tomato TOP3α protein is 915 amino acids (aa) in length and contains the domains known to be important for the function of TOP3α in *A. thaliana* (Dorn et al. 2018); N-terminal TOPRIM domain, central domain with the active site (red line) and C-terminal zinc (Zn) finger domains T1, two CCHC-type and GRF-type, the same as with Arabidopsis.
(b) *SlTOP3α* gene structure indicating exons (boxes) and domain structure within these. Black line represents the untranslated regions, including introns. The *TOP3α* gene in tomato is comprised of 24 exons and is 3564 bp in length. CRISPR/Cas9 cutting site within exon 1 is indicated by the arrow.

but unique role elucidated during late meiotic recombination for RMI1, as part of a subcomplex with TOP3α, was thought to be representative of a plant-specific role. However, supporting the above findings that tomato *rmi1* mutants are fully fertile, 4′,6-diamidino-2-phenylindole (DAPI)-staining of chromatin also confirmed that meiotic

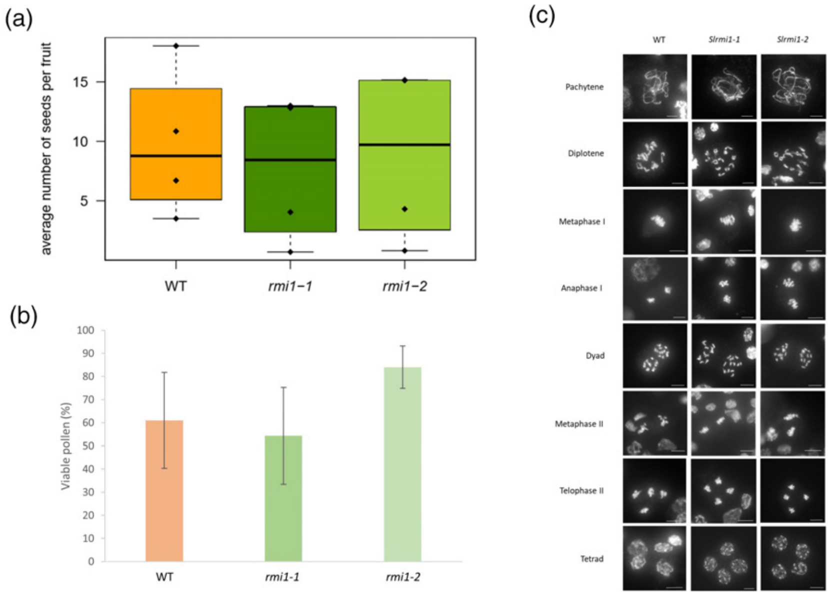

Figure 3. Fertility analysis of *rmi1* homozygous tomato mutant lines.

(a) Boxplot illustrating the average number of seeds per ripe fruit from mature *rmi1-1* and *rmi1-2* mutant plants, compared with wild-type (WT) plants of the same age, determined from four independent assays with >10 fruits analysed from five plants, for each line. Median for the WT line and two *rmi1* mutant lines, indicated by the black lines, and interquartile range as shown by the coloured boxes, are similar for each line, indicating that there is no reduction in the seed number per fruit for both mutant lines, *rmi1-1* and *rmi-2*, compared with WT. *P*-values were calculated according to the Mann–Whitney *U*-test, which confirmed that there was no statistical difference between the lines. Individual data points for the assays are shown as black diamonds.

(b) Percentage of viable pollen determined via fluorescein diacetate staining of spores for both *rmi1-1* and *rmi1-2* and WT as control, from three independent assays. Pollen from one flower bud per line was used for each assay. Mean ± standard deviation (error bars) was determined and statistical analysis was calculated using a two-tailed *t*-test with unequal variances. Both *rmi1* mutant lines show no statistically significant difference in the percentage of viable pollen to WT.

(c) DAPI-stained chromatin spreads of tomato pollen mother cells from WT and the homozygous mutant lines *rmi1-1* and *rmi1-2*. Compared with WT, the two *rmi1* lines did not show any increased number of defects during meiotic progression, with both meiosis I and meiosis II appearing normal with the correct formation of dyads and tetrads, respectively. Scale bars represent 10 μm.

progression is normal when compared with that of WT (Figure 3c). Taken together, the fertility analyses carried out with both mutant lines suggest that RMI1 may not be an essential factor of meiotic recombination in tomato, standing in contrast to the observed role in Arabidopsis.

RMI1 is not required for DNA repair in tomato

With it being seemingly, but surprisingly evident that RMI1 has no meiotic role in tomato, in stark contrast to Arabidopsis (Chelysheva *et al*., 2008; Hartung *et al*., 2008), we were interested in the role of SlRMI1 regarding somatic DNA repair. Therefore, we investigated the DNA repair capacity of the *rmi1* mutant lines with regard to a variety of genotoxic agents. A liquid assay was carried out whereby the weight of 15-day-old seedlings was measured following treatment with the DNA damaging agents for 6 days, in relation to an untreated control. Interestingly, neither *rmi1-1* nor *rmi1-2* showed increased sensitivities when subjected to the crosslinking agent cisplatin or the alkylating agent methylmethane sulfonate, when compared with the WT control (Figure 4a,b). Furthermore, both *rmi1* tomato mutant lines did not show any enhanced sensitivity when treated with the topoisomerase 1 inhibitor camptothecin or the crosslinker mitomycin C (Figure 4c,d). In addition, we tested whether the expression of Sl*RMI1* is induced by treatment with the DNA cross-linking agent cisplatin (Figure S3, Method S1). Similar to the situation in Arabidopsis, the expression level of both Sl*RMI1* and Sl*TOP3α* stays in the same range as without cisplatin treatment. In contrast to this, the level of Sl*BRCA1* expression, serving as an internal control, was clearly upregulated following cisplatin treatment (Chen *et al*., 2003). Consequently, it appears that SLRMI1 plays at least no significant role in response to DNA damage induced by genotoxins.

SLRMI1 is not involved in the repair of replication-associated DNA-repair processes

With no apparent role for RMI1 in tomato in somatic DNA repair following genotoxin-induced damages, we analysed further whether it might be involved in replication-associated repair processes. Therefore, we investigated cell division in dividing tissues within the root meristem by conducting root length and cell viability analyses. The accumulation of spontaneous DNA damage can lead to cell death, and damage within dividing cells can result in decreased root lengths (Beemster and Baskin, 1998). Thus,

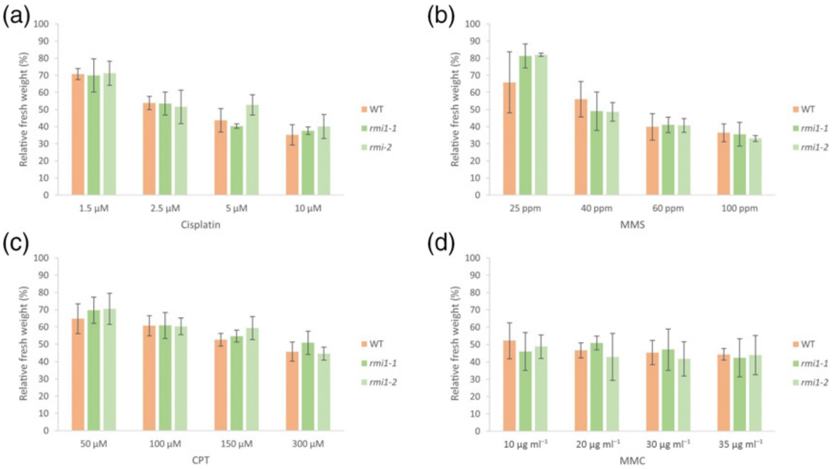

Figure 4. Genotoxin sensitivity analysis of tomato *rmi1* mutant lines.

Mean values of seedling fresh weight of the *rmi1* mutant lines, *rmi1-1* and *rmi1-2*, and wild type (WT), relative to untreated controls following treatment with genotoxic agents. Standard deviation (error bars) was calculated and statistical analysis was determined using a two-tailed *t*-test with unequal variances.

(a) Mean relative fresh weight of seedlings following treatment with 1.5, 2.5, 5 and 10 µM cisplatin (n = 3). Both *rmi1* mutant lines did not show a reduced relative fresh weight at any of the concentrations used, compared with WT.

(b) Mean relative fresh weight of seedlings following treatment with 25 parts per million (ppm), 40, 60 and 100 ppm methylmethane sulfonate (MMS) (n = 3). *rmi1-1* nor *rmi1-2* did not show a reduced relative fresh weight at any of the concentrations used compared with WT, indicating no sensitivity.

(c) Mean relative fresh weight of seedlings following treatment with 50, 100, 150 and 300 µM camptothecin (CPT) (n = 3). Both *rmi1* mutant lines did not show a reduced relative fresh weight at any of the concentrations used, compared with WT.

(d) Mean relative fresh weight of seedlings following treatment with 10, 20, 30 and 35 µg ml^{-1} mitomycin C (MMC) (n = 3). Both homozygous *rmi1* mutant lines did not show a sensitivity via reduced fresh weight, at any of the concentrations used, compared with WT.

analysis of the root meristem is beneficial for gaining an insight into the involvement of factors in replication-associated DNA repair. We analysed the root lengths of the two *rmi1* tomato mutant lines, with respect to WT. Plants deficient in RMI1 did not show significantly reduced root lengths compared with WT, showing no defects in root growth (Figure 5a). Additionally, the azo dye Evan's blue was used to quantify cell viability within the roots. Evan's blue is able to penetrate damaged membranes, staining cells. The uptake of the dye can then be measured to determine cell viability (Nv *et al*., 2017). We measured the uptake of Evan's blue dye for both *rmi1* mutant lines and a WT control. With no difference between the uptake of Evan's blue between the mutant lines and WT (Figure 5b), it appears that cell viability is not influenced when plants are lacking RMI1. As a result, RMI1 does not appear to be involved in replication-associated DNA repair in tomato, providing further evidence that RMI1 is not required in tomato for somatic DNA repair processes.

TOP3α mutants in tomato are embryo-lethal

With the inclination that RMI1 may not be involved in either somatic DNA repair or meiotic processes in tomato, we were intrigued to determine whether the role of TOP3α also differs to that known for Arabidopsis, where the RTR complex partner plays a significant role in plant DNA repair and meiosis (Dorn *et al*., 2018; Hartung *et al*., 2008). Following Cas9-mediated mutagenesis, two different heterozygous mutant plants were identified within the T1 generation, the first with a 1-bp insertion and the second with a 4-bp deletion, both within the first exon of *TOP3α*. Both mutations were confirmed on an mRNA level via Sanger sequencing, and resulted in premature stop codons following frameshifts within the open reading frames (Figure S4). Both heterozygous mutant plants were grown to maturity, and fruits of the T2 generation were harvested for seed collection. Seeds were collected using a 1 mm mesh sieve, sterilized and sown on to germination medium. Following 2 weeks of growth in a growth chamber, DNA was extracted and used for HRM and Sanger sequencing analysis to identify plants with a mutation on both alleles. For both independent mutant lines, named *Sltop3α-1* and *Sltop3α-2*, respectively, no homozygous plants could be identified. When considering Mendelian genetics, 25% of all progeny from a heterozygous self-pollinated plant should be homozygous. However, from all plants analysed, for both lines, around a third were found to be WT, with no mutations on either alleles, and the remaining two-thirds were heterozygous (Table S2). With these data, we were led to believe that the homozygous *top3α* mutant plants might not be viable.

Both *top3α* heterozygous lines, *Sltop3α-1* and *Sltop3α-2*, showed a significantly reduced number of seeds compared with WT, following seed collection using a 1-mm mesh sieve, with about 25% less seeds relative to the WT control (Figure 6a). Despite this, the heterozygous plants did not seem to have any other apparent defects regarding somatic growth and fruit size or number (Figure S5a,b,d).

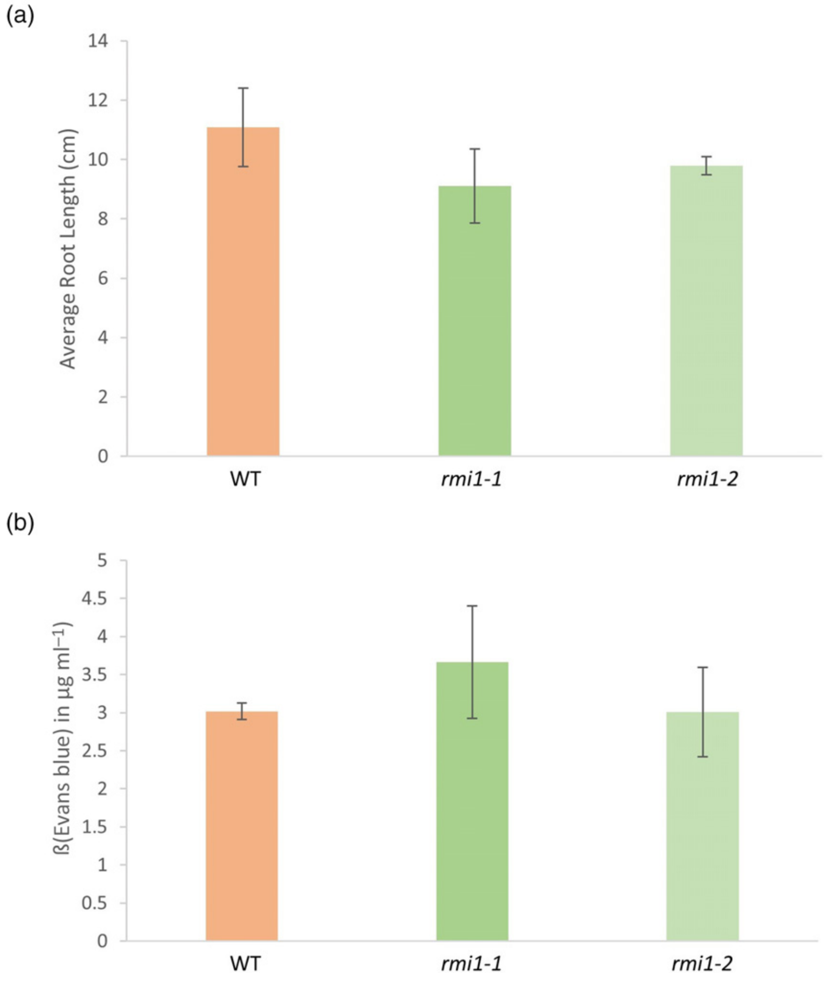

Figure 5. Root length and meristematic root cell viability analysis in *rmi1* tomato mutant lines.
(a) Mean values of root length (five roots) measured from 12-day-old seedlings of both *rmi1-1* and *rmi1-2* mutant lines, compared with wild type (WT) (n = 3). Error bars correspond to standard deviation. Statistical analysis was determined using a two-tailed t-test with unequal variances. Average root length of both *rmi1* mutant lines was comparable with that of the WT control.
(b) Mean measurement of Evan's blue uptake (μg ml^{-1}) from five roots of 9-day-old seedlings for *rmi1-1* and *rmi1-2* mutant lines, compared with WT (n = 3). Error bars correspond to standard deviation. Statistical analysis was determined using a two-tailed t-test with unequal variances. Evan's blue uptake was similar for all lines, suggesting no difference in cell viability between the two *rmi1* mutant lines and the WT control.

FDA staining was also carried out whereby pollen viability was not shown to be different to WT (Figure S5c). However, upon dissection of fruits it was noticeable that a number of small seeds (<1 mm) were visible within the *top3α* lines, more than for the WT control line (Figure 6b). These small seeds had not been collected beforehand due to the width of the sieve openings used for collection. Therefore, we proposed that these small seeds could account for the progeny with the homozygous mutations. If this were to be the case, following Mendelian segregation, these small seeds would represent ¼ of all the seeds obtained from heterozygous plants. In order to test this hypothesis, both regular and small seeds were counted for *Sltop3α-1*, *Sltop3α-2* and a transformed WT line of the same generation that had been subjected to the same growth conditions, as an appropriate control. In relation to the WT control, with 100% regular seeds, approximately 25% of the total number of seeds from both *top3α* heterozygous lines were small (<1 mm), with approximately 75% being regular seeds (>1 mm) (Figure 6c). A χ^2-test was carried out to clarify our hypothesis, the results of which verified that the ratio of small seeds corresponded to a quarter of all seeds, for both lines (Table S3). As the small seeds were not able to germinate, they were deemed non-viable. Thus, if these small seeds are indeed the homozygous null *top3α* mutants as proposed, then lack of TOP3α in tomato could lead to non-viable plants with a plausible explanation being defects with embryo development. Embryo lethality in tomato *top3α* mutants hints that TOP3α plays an essential role in tomato, which is very surprising considering the

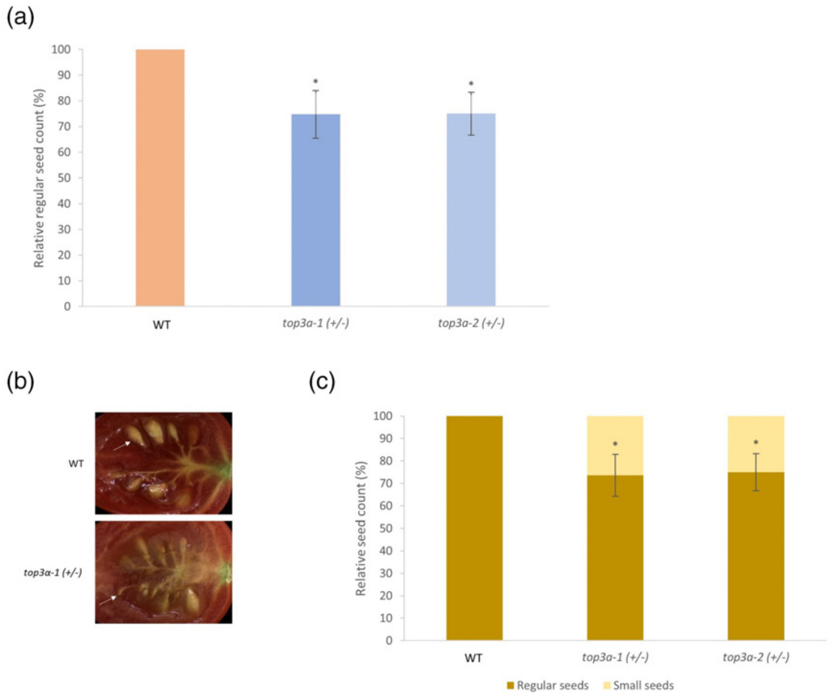

Figure 6. Analysis of heterozygous *top3α* tomato lines.

(a) Relative regular (>1 mm) seed number from *top3α-1* and *top3α-2* heterozygous plants, and the respective wild-type (WT) control plants. Columns correspond to mean values (n = 3) and error bars represent ± standard deviation. Regular seeds of >10 ripe fruits were counted from 10 plants, for each independent assay. Statistical differences were calculated using a two-tailed t-test with unequal variances: $*P < 0.05$. Both *top3α* lines show a significantly reduced number of regular seeds relative to that of the WT line, with a reduction of about 25%.

(b) Photograph highlighting an exemplary regular seed (arrow) of >1 mm in WT and a small seed (arrow) of <1 mm in the *top3α-1* (±) line.

(c) Relative total seed numbers of both regular and small seeds from both *top3α-1*, *top3α-2* heterozygous plants and the respective WT control plants. Columns correspond to mean values (n = 3) and error bars represent ± standard deviation. Regular seeds of >10 ripe fruits were counted from 10 plants, for each independent assay. Statistical differences were calculated using a two-tailed t-test with unequal variances: $*P < 0.05$. Both *top3α* lines have about 25% more small seeds, relative to the WT control.

viability of the *top3α* Arabidopsis mutants thought to be representative of all plants (Dorn *et al*., 2018).

DISCUSSION

The RTR complex has been studied extensively, with both RMI1 and TOP3α demonstrating integral roles in genome stability in a number of eukaryotic organisms including yeast, animals and plants. Although there are slight differences between kingdoms regarding the extent of these roles, the consensus is that both RMI1 and TOP3α are essential for DNA repair and HR, due to their fundamental roles in the dissolution of HJ-like recombination intermediates. For the plant model organism, *A. thaliana*, important functions in recombination suppression and the repair of a multitude of DNA lesions were demonstrated for both RMI1 and TOP3α, as also observed for yeast and animals (Dorn *et al*., 2018; Hartung *et al*., 2008). Yet, unlike for various animals whereby lack of TOP3α leads to embryo lethality, studies in Arabidopsis revealed this not to be the case in plants, with mutants being viable (Dorn *et al*., 2018). In addition to this, a unique role in meiosis was also shown for both RMI1 and TOP3α in plants, with Arabidopsis mutants showing severe meiotic defects leading to sterility (Chelysheva *et al*., 2008; Hartung *et al*., 2008). However, if these remarkable peculiarities are general features for plant RMI1 and TOP3α or just apply to Arabidopsis, was unclear until now. In this study, using Cas9-mediated mutagenesis to generate *rmi1* and *top3α* mutant lines in tomato, we aimed to clarify the role for both genes in dicotyledonous plants and thereby revealed dramatically differing functions to those found for the Arabidopsis homologues.

Differences in functions of RMI1 between tomato and Arabidopsis

First, upon establishing two independent *rmi1* mutant lines in tomato, *Slrmi1-1* and *Slrmi1-2*, it was apparent that homozygous mutants are fully fertile. The fertility of the mutants was not different to that of the WT control plants upon analysis of seed number (Figure 3). This was a surprise considering the sterility of the Arabidopsis *rmi1* mutants with drastic meiotic defects leading to an arrest of meiotic division after meiosis I (Chelysheva *et al*., 2008; Hartung *et al*., 2008). However, analysis of meiotic progression in tomato *rmi1* mutants did not show any defects, with normal progression throughout meiosis I and II. The viability of pollen was also shown not to be affected, with no visibly increased number of dead pollen compared with the WT control. Taking all of this into account, tomato *rmi1* mutants seem to be fully fertile without any restrictions. Therefore, this was the first hint to a fundamental difference between tomato and Arabidopsis homologues, as RMI1 in tomato does not seem to have a role in meiosis, let alone an essential one, as is the case for Arabidopsis.

As no meiotic role for RMI1 in tomato could be elucidated in this study, we were interested if the homologue would play a role in somatic DNA repair. However, treatment of tomato mutants with genotoxic agents showed no apparent sensitivity to any of the genotoxins tested (Figure 4), as opposed to Arabidopsis *rmi1* mutants displaying increased sensitivity to both the crosslinking agent cisplatin and the alkylating agent methylmethane sulfonate (Hartung *et al*., 2008). With these results, it seems that RMI1 may not be required for the repair of these certain kinds of induced DNA damages in tomato. Moreover, analysis of root length and cell viability within the root meristem suggests that not only is RMI1 apparently not involved in somatic DNA repair in tomato; however, it is also not required for replication-associated repair processes (Figure 5). This is the first indication that RMI1, when present, may not in any case have a significant role in DNA repair in all eukaryotes, but also implies stark differences to what had been found for Arabidopsis and was previously considered representative of all plants.

Thus, the role of RMI1 in tomato does seem to differ despite the homologue being similar on a sequence level to that of Arabidopsis, and having the conserved functional protein domains, DUF1767 and OB1-fold domain, known to be essential for both the somatic and meiotic roles of AtRMI1 (Figure 1a). The tomato RMI1 homologue also has the OB2-fold domain, shown to enable the interaction with TOP3α in Arabidopsis (Bonnet *et al*., 2013).

TOP3A has an essential role in tomato

RMI1 and TOP3α form an interacting subcomplex that mediates the dissolution of recombination intermediates during somatic DNA repair in most organisms. Therefore, we were intrigued to elucidate whether TOP3α is required for DNA repair in tomato. TOP3α is essential in mammals with mutants being completely embryo-lethal (Li and Wang, 1998). Severe developmental defects were also demonstrated for both *C. elegans* and *D. melanogaste*r, illustrating the essential role of TOP3α for various animals (Kim *et al*., 2000; Plank *et al*., 2005). Although unclear in Arabidopsis for a number of years, Dorn et al. (2018) recently revealed that *top3a* mutants in Arabidopsis are viable, albeit mutant plants display a number of drastic defects, including meiotic catastrophe owing to the significant role of TOP3α in plant DNA repair and meiotic recombination. The results we obtained in this study indicated that *top3a* tomato mutants are actually embryo-lethal, similar to the situation in mammals. No homozygous mutant plants were identified from both established mutant lines, only heterozygous and WT, which accounted for about two-thirds and a third of the plants analysed, respectively. Interestingly, small seeds were visibly more notable in the fruits of the *top3a (+/–)* lines and their quantification showed that these account for the 25% reduction in regular seeds for *top3a (+/–)*, compared with WT (Figure 6). Considering Mendelian segregation, the progeny of a heterozygous self-pollinating mutant plant would be comprised of 50% heterozygous individuals, 25% WT and 25% homozygous plants. From our results, we deduced that the small seeds observed for the *top3a (+/–)* lines correspond to the homozygous mutant plants (Table S3). As these small seeds were not viable, it seems plausible to suggest that there is some defect during embryo development, which leads to embryo lethality. With what is known from the biochemical functions of TOP3α homologues, the absence of SlTOP3α might result in the accumulation of unresolved aberrant replication intermediates, ultimately leading to cell death. Thus, our results suggest the TOP3α of tomato is as essential as in animals for maintaining genome stability.

Differential functions of RTR complex partners between dicotyledonous species

As we were not able to demonstrate any role for SlRMI1 in somatic or meiotic DNA repair, this raises the question as to whether other factors are able to replace it in tomato functionally. One has to keep in mind that RMI1 itself has no biochemical activity for DNA processing but is merely stabilizing the RTR complex. Certainly, examples of life without RMI1 can be found in other eukaryotes. *Drosophila* is lacking an RMI1 homologue, which seems to be substituted by an insertion within the C-terminus of DmTOP3α (Chen *et al*., 2012). Thus, it seems possible that the key role in stabilization of the RTR complex in tomato might be taken over by other factors.

Recent findings hint to a possible candidate in substituting for the role of RMI1 in tomato. In rice, a new protein associated with TOP3α was found, which also has a strong meiotic phenotype. Mutants of meiotic chromosome association 1 (MEICA1) are almost completely sterile, with chromosome bridges and extensive fragmentation during anaphase I of meiosis. Interestingly, these defects are reminiscent of both the Arabidopsis *top3α* and *rmi1* mutant meiotic phenotypes. MEICA1 is carrying a DUF4487 domain, which has no clearly defined molecular function but is highly conserved throughout MEICA1 homologues. Rice MEICA1 was speculated to act as a possible partner of

TOP3α in meiotic joint molecule intermediate processing (Hu *et al.*, 2017).

Quite recently, the Arabidopsis homologue of MEICA1, the FIDGETIN-LIKE-1 INTERACTING PROTEIN (FLIP) was characterized and shown to form a complex with the meiotic CO-limiting factor FIDGETIN-LIKE 1 (FIGL1), with both factors acting in the same anti-CO-limiting pathway (Fernandes, Duhamel *et al.*, 2018). Nevertheless, the mutants of rice and Arabidopsis differ drastically in their meiotic phenotype: for *flip* mutants in Arabidopsis, a slight increase in CO frequency was demonstrated, although meiosis progresses relatively normally with only minor defects.

We identified the putative homologue of AtFLIP/OsMEICA1 within the tomato genome and could confirm that it also has the conserved DUF4487 domain (Figure S6). With no apparent functional role for RMI1 in tomato, and the strongly differing functions of MEICA1 between species, we are tempted to speculate that MEICA1 and RMI1 homologues in their interaction with TOP3α are of contrasting importance in different plant species with stabilization of the RTR complex. Whereas RMI1 is essential for proper meiotic chromosome segregation in Arabidopsis, the MEICA1 function is not as essential as in other plants. We speculate that *meica1* mutants of tomato share similar meiotic phenotypes and display somatic defects as in rice, suggesting a role for MEICA1 in meiotic recombination and somatic DNA repair as a substitute for RMI1. Therefore, it would also be interesting to determine the phenotype of the *Slmeica1*, as well as the *Osrmi1* mutant.

While providing an insight into the functional roles of both the RTR complex partners TOP3α and RMI1 in tomato, we have now also provided evidence that there are differences between plant species, and even between dicotyledons. With increasing numbers of studies being carried out in crop plants over recent years, the phenomenon of such differences between Arabidopsis, the model plant species, and other species has become a reoccurring issue, also apparent with the differences found for AtFLIP/OsMEICA1. Previously, studies were routinely carried out in Arabidopsis due to its advantageous characteristics as a model organism, such as its small size, fast generation time and the vast genomic resources available. However, due to the need to improve crop breeding to aid with efforts to ensure food security, much of the knowledge gained from these studies is now being translated to crop species to find ways to manipulate meiotic recombination to accelerate plant breeding efforts. The recent findings of Maagd *et al.* (2020) are also an example of differences between Arabidopsis and tomato concerning the third RTR complex partner RECQ4. Maagd *et al.* (2020) generated a biallelic *recq4* interspecific tomato mutant, which showed a 1.53-fold increase in COs when analysing ring bivalents. Although very promising in itself, it is strikingly less than the six- and four-fold increase observed for Arabidopsis Col-0 *recq4a/recq4b* double mutants and Col-0/Ler hybrids, respectively (Fernandes, Séguéla-Arnaud *et al.*, 2018), hinting at a less important role in negatively regulating COs for tomato RECQ4 than for Arabidopsis. As an RTR complex partner alongside RMI1 and TOP3α, these recent findings regarding RECQ4 are interesting as it supports our findings that the roles of these factors may differ between plant species. Our results show that it is advisory to disregard previous findings from Arabidopsis as generally being representative of all plants, for both dicots and monocots. The differences between plant species, hinting at plant specific roles for a number of factors, act to strengthen the notion that the translation of knowledge from Arabidopsis to crops is not as straight forward as first preconceived, and thus further research, such as this present study, using non-model crop species needs to be conducted for the plant-specific development of strategic breeding approaches. The rise of the CRISPR/Cas technology now finally allows us to obtain mutants in non-model crops with ease (Schindele *et al.*, 2020; Zhu *et al.*, 2020).

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Solanum lycopersicum cultivar Micro-Tom (MT) (Campos *et al.*, 2010), ecotype WT-BDX (obtained from Christophe Rothan, INRA, France) was used for this study. Plants were grown in the greenhouse on substrate containing 1:1 mixture of Floraton 3 (Floragard, Oldenburg, Germany) and vermiculite (2–3 mm; Deutsche Vermiculite Dämmstoff, Sprockhövel, Germany), at 24°C during the day and 20°C at night, with alternating 16 h light and 8 h darkness. For *in vitro* cultivation, plants were grown in a growth chamber in an ECO2BOX with green filter (Duchefa, Biochemie, Haarlem, The Netherlands) containing germination medium (GM: 2.17 g l^{-1} Nitsch 224 (Duchefa, Biochemie, Haarlem, The Netherlands), 20 g l^{-1} saccharose, pH 5.8, 9 g l^{-1} micro agar (Duchefa, Biochemie). Plants grown in the growth chamber were subjected to 14 h light and 10 h darkness, under axenic conditions.

Agrobacterium-mediated transformation and CRISPR/Cas9-mediated mutagenesis

To generate the mutant lines analysed in this study, cotyledons of Micro-Tom (WT-BDX) seedlings were transformed using the Agrobacterium strain GV3101, as described in Meissner et al. (1997) with the same modifications as discussed in Dahan-Meir et al. (2018), harbouring the CRISPR/Cas9 constructs using the Cas9 from *Streptococcus pyogenes*, as previously described (Fauser *et al.*, 2014). The phosphinothricin resistance cassette in the pDe-SpCas9 destination vector had been substituted with a kanamycin resistance cassette using the restriction enzyme *Hin*dIII. Transgenic T0 plants were selected using kanamycin resistance. Single locus lines were identified in the T1 generation by a 3:1 Mendelian segregation of the kanamycin resistance cassette, using polymerase chain reaction (PCR) amplification. HRM analysis and Sanger sequencing were used to validate the zygosity of the induced mutations in the T2 generation, and PCR screening of the Cas9 was carried out to ensure plants were free of the transgene. Suitable lines were further propagated to obtain seeds for experimental use. For characterization of TOP3α and RMI1 in tomato, the respective tomato lines *top3α-1*, *top3a-2* and *rmi1-1*, *rmi1-2* were established and the genotypes were confirmed via PCR and Sanger sequencing (Table S1).

Pollen viability analysis via FDA staining

FDA staining was performed as described by Heslop-Harrison and Heslop-Harrison (1970). A stock solution of 2 mg ml^{-1} FDA was prepared in acetone and diluted to 100 μl ml^{-1} with 7% sucrose in water (w/v) to prepare the working staining solution. Mature flower buds were transferred into a tube with 350 ml staining solution and then vortexed for 5 min. Flowers were removed and the solution was centrifuged (5 min at 10 000 ***g***). The stained pollen pellet was washed with 7% sucrose in distilled water and a drop was transferred to a microscope slide. The slide was incubated in the dark for 10 min and the stained pollen was visualized with a fluorescence binocular microscope.

Meiotic chromosome behaviour analysis via DAPI staining of male meiocytes

Chromatin preparations of male meiocytes were performed as described by Armstrong et al. (2009), with the following modifications. The buds were digested for an extended period of 4 h and the petals and sepals were removed before bud preparation on a microscope slide.

Sensitivity assays

Assays to determine sensitivity against genotoxins were carried out as previously described (Hartung *et al.*, 2007), with optimization for tomato. Five 9-day-old plantlets grown in axenic conditions were transferred into one well of a six-well plate containing 4 ml liquid GM. After 24 h, 1 ml of genotoxin solution was added to obtain the desired concentration in a total volume of 5 ml, with only 1 ml GM liquid medium being added for the untreated controls. The fresh weight of the plants were measured after 6 days in a growth chamber, and normalized to the respective untreated controls to obtain the relative fresh weight.

Root length analysis

Plantlets grown in the growth chamber for 12 days were gently placed on to a black piece of card. A clear plastic tray was used to apply gentle pressure on top of the plants and photos were taken. The length of the roots were determined using the SmartRoot Plug-In of ImageJ (Lobet *et al.*, 2011).

Cell viability quantification

Cell viability was quantified based on the Evan's blue staining procedure described by Nv et al. (2017). Nine-day-old plantlets grown under sterile conditions were carefully removed from solid GM and 1 cm of root from five plants, including tips, were excised and placed into a tube. The excised roots were incubated in a 0.25% solution of Evan's blue for 20 min at room temperature, with shaking at 300 rpm. The root material was washed three times in distilled water and homogenized in 1 ml 1% sodium dodecyl sulphate. After 5 min centrifugation at 12 000 ***g***, the supernatant was transferred to a new tube and the optical density was measured at 600 nm. The absorbance obtained was compared with a standard curve previously prepared, to determine the amount of Evan's blue in the sample.

Bioinformatics

Homology searches were completed NCBI BLAST (Altschul *et al.*, 1990). Multiple sequence alignments were performed using Clustal Omega (Sievers *et al.*, 2011). Protein domain analysis was performed using EMBL-EBI InterProScan 5 (Madeira *et al.*, 2019). Phylogenetic trees were constructed using iTOL v5 (Letunic and Bork, 2019). Gene structure was determined using NCBI Splign (Kapustin *et al.*, 2008).

Statistical methods

Analysis to determine statistical significance of data was carried out using a two-sided, two-sample *t*-test with no equal variance. $P < 0.05$ was statistically significant.

Accession numbers

Sequence data from this article can be found with the following locus identifiers within the current Tomato Genome version SL4.0 available from The Sol Genomics Network (SGN): *SlTOP3α*, Solyc05g014720.3; *SlRMI1*, Solyc12g005900.2; *SlRMI2*, Solyc11g066690.2; and *SlMEICA1*, Solyc09g091370.3 (Fernandez-Pozo *et al.*, 2015). MEICA1/FLIP orthologue data can be found using the following identifiers: *Medicago truncatula*, XP_003591635.2; *Glycine max*, XP_014627763.1; *Vitis vinifera*, XP_019075832.1; *Arabidopsis thaliana*, AT1G04650; *Brassica rapa*, XP_009111092.1; Homo sapiens NP_060656.2; *Physcomitrella patens*, XP_001766106.1; *Zea mays*, XP_008650960.1; *Sorghum bicolor*, XP_002465870.1; *Hordeum vulgare*, KAE8799555.1.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

AW, AD, SR and HP designed research; AW performed research; AW, AD and HP analysed data; and AW, AD and HP formulated the manuscript.

DATA AVAILABILITY STATEMENT

The data obtained and material used in this study, including plant lines and constructs, can be obtained upon request via email with the corresponding authors.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *SlRMI1* cDNA sequence alignment.

Figure S2. *Slrmi1-1* and *Slrmi1-2* homozygous mutant plants.

Figure S3. Relative expression level of RMI1, TOP3α and BRCA1 in *Arabidopsis thaliana* and *Solanum lycopersicum* after cisplatin-treatment.

Figure S4. *SlTOP3α* cDNA sequence alignment.

Figure S5. *SlTOP3α* heterozygous mutant analysis.

Figure S6. OsMEICA1/AtFLIP phylogenetic analysis and domain structure comparison.

Table S1. Oligonucleotides used.

Table S2. Number and percentage of heterozygous and homozygous mutant plants and wild-type (WT) plants, identified from the progeny of two heterozygous *top3α* lines, *top3α-1* and *top3α-2*.

Table S3. Percentage of regular and small seeds in both *top3α* heterozygous lines, compared with the expected when the small seeds correspond to the homozygous progeny and the regular seeds account for WT and heterozygous.

Methods S1. qRT-PCR analysis of *RMI1*, *TOP3α* and *BRCA1* gene expression.

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