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Meiotic recombination profiling of interspecific hybrid F1 tomato pollen by linked read sequencing

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SUMMARY

Genome wide screening of pooled pollen samples from a single interspecific F1 hybrid obtained from a cross between tomato, *Solanum lycopersicum* and its wild relative, *Solanum pimpinellifolium* using linked read sequencing of the haploid nuclei, allowed profiling of the crossover (CO) and gene conversion (GC) landscape. We observed a striking overlap between cold regions of CO in the male gametes and our previously established F6 recombinant inbred lines (RILs) population. COs were overrepresented in non-coding regions in the gene promoter and 5'UTR regions of genes. Poly-A/T and AT rich motifs were found enriched in 1 kb promoter regions flanking the CO sites. Non-crossover associated allelic and ectopic GCs were detected in most chromosomes, confirming that besides CO, GC represents also a source for genetic diversity and genome plasticity in tomato. Furthermore, we identified processed break junctions pointing at the involvement of both homology directed and non-homology directed repair pathways, suggesting a recombination machinery in tomato that is more complex than currently anticipated.

Keywords: recombination bin mapping, tomato, crossover recombination, gene conversion, *S. pimpinellifolium*, recombination hot spots, recombination cold spots.

INTRODUCTION

Meiotic recombination is a key fundamental biological process that ensures balanced chromosome distribution and the reshuffling of parental alleles into new combinations. In the current model, meiotic recombination is initiated by the formation of double-stranded DNA breaks (DSBs) that are processed to single stranded DNA, followed by single strand invasion into the intact sister chromatid or one of its non-sister chromatids. Upon break repair on the non-sister chromatids, DSBs are resolved either into crossovers (COs) or non-crossovers (NCOs). The resolution involves homology directed repair (HDR) by the ZMM and non-

ZMM pathways leading to class I and class II COs respectively, and alternative pathways such as synthesis-dependent strand annealing (SDSA), dissolution of double Holiday junction (dHJ) and other pathways leading to NCOs (Mercier *et al.*, 2015). In general, CO involves reciprocal exchange of large DNA fragments, which can size up to whole chromosome arms. In contrast, a NCO usually comprises a small patch that is copied from the intact homologous chromosome (Mercier *et al.*, 2015). When the sister chromatid template is used as donor, the repair machinery restores the duplex. When the non-sister chromatid

template is used as a donor, sequence divergence at the site of strand invasion may give rise to heteroduplex formation. The mismatch repair machinery recognizes and corrects meiotic heteroduplex DNA (Borts *et al.*, 2000), leading to a non-reciprocal exchange of genetic information or gene conversion (GC). Gene conversion can occur between donor and acceptor alleles of the same gene (allelic GC) or between different repetitive loci, the latter known as ectopic gene conversion (non-allelic GC). (Puchta, 2005; Duret and Galtier, 2009; Wijnker *et al.*, 2013; Trombetta *et al.*, 2016).

In plant breeding, the allelic reshuffling and gene conversion via meiotic recombination is a source of genetic diversity exploited by breeders that seek out new allele combinations for trait selection and crop improvement. As assorted alleles determine the inherent properties in progeny, it is important to precisely introgress chromosomal regions of interest, maintaining favorable genes yet preventing other regions harboring undesirable alleles to be recombined. Detection of genetic differences and monitoring of specific characters in segregating populations to assist trait selection is therefore crucial to successful breeding. Efficient detection and precise delineation of recombined alleles has seen many strategies. Microscopic analysis of meiotic events in tomato has provided valuable information on chromosome configurations (Moens, 1964; Havekes *et al.*, 1994; Anderson *et al.*, 2010a,b), but the reduced throughput and low resolution limits genome wide and accurate measurements of recombined alleles. Currently, prevailing technologies to profile segregating alleles in populations include molecular marker-based screenings. In combination with high-throughput genome sequencing, such technologies are extremely powerful to delineate recombined alleles. Furthermore, such screenings provide valuable information on recombination frequency and genetic distances, which is used for genetic linkage map construction and marker assisted breeding. Previously, we profiled recombination events in a RIL population from a cross between tomato and its close relative *S. pimpinellifolium*, using genome wide SNP analysis. The analysis provided insight into 'hot' and 'cold' regions of recombination, and genomic features involved in crossover recombination (Aflitos *et al.*, 2015; Demirci *et al.*, 2017, 2018). However, these methods require the production of offspring populations involving several generations and subsequent genetic marker screening for a large number of progeny plants (Paran *et al.*, 2004; Van Os *et al.*, 2006; Ganai *et al.*, 2011; Baurens *et al.*, 2019). Often marker coverage is limited, causing low genetic resolution, and when integrated with physical maps, allows detection of crossovers in the kilo base range at best. Markers may even be absent leaving substantial genome portions uncovered and thus opaque to further genetic profiling. Furthermore, the accurate detection of COs and GCs by

NGS technologies has long been hampered by short read length limitations, a relatively high base call error rate for long reads, and/or inadequate bioinformatics solutions. Therefore, more affordable quantitative methods to study CO and GC distributions are desired.

Here we present a method for profiling meiotic recombination in pollen nuclei from a single F1 interspecific hybrid between tomato and the closely related *S. pimpinellifolium*. The identification of polymorphisms marking COs and GCs is greatly improved by high-throughput long read sequencing with high base call accuracy, facilitating unambiguous read mapping and accurate identification of haplotype phases. We pinpoint COs and GCs at the SNP resolution level, using 10X Genomics linked read sequencing of gDNA from F1 pollen nuclei and subsequent detection of phase shifts in large gDNA molecules, providing further insight into gross similarities of recombination. Our sequence based approach can be applied to reliably profile meiotic recombination in a wide variety of crop species without the laborious and time-consuming production and screening of offspring populations, greatly benefitting introgression hybridization and precision breeding.

RESULTS

Detection of haplotype shifts and CO designation

Our aim was to localize recombination events at SNP resolution, taking advantage of linked-read sequencing of an F1 hybrid pollen grain population and the high density of informative polymorphisms between *S. lycopersicum* Heinz 1707 and *S. pimpinellifolium* CGN14498 parental genomes, allowing a detailed analysis of genomic sequences involved in CO recombination. To this end we constructed a linked read sequencing library from ~0.70 ng input gDNA and produced 781.3×10^6 paired end (PE) linked reads. With a median insert size of 391 bp, the data represented a mapped genome coverage of about 68×. Approximately 60% and 18% of the input gDNA molecules was longer than 20 and 100 kb, respectively, with a mean molecule length of 25.5 kb. We obtained 1.61×10^6 gel beads in emulsion (GEMS) with a mean DNA molecule length per GEM of 285 kbp, and an N50 linked reads per molecule (LPM) of 12. Given these GEM performance statistics, the molecule coverage for each genome copy was approximately 86%. Using the LONG RANGER pipeline from 10X Genomics, approximately 95.7% of the reads was successfully mapped to the tomato reference genome, leaving less than 0.14% of the reference bases uncovered. We detected polymorphisms in *S. pimpinellifolium* and *S. lycopersicum* PE reads that were aligned to the tomato SL3.0 reference genome, using SNP callers from CLC and GATK (McKenna *et al.*, 2010). Approximately 4.65×10^6 CLC and 4.90×10^6 GATK *S. pimpinellifolium* homozygous SNP calls were intersected, based on their corresponding

genomic position, rendering 4.05×10^6 high quality homozygous SNPs (Figure S3). To remove possible base call errors and individual-specific sequence differences, approximately 210 000 SNPs between the tomato parent and the reference genome were excluded, leaving 3.84×10^6 homozygous SNPs at a SNP density level of 1 per 210 bp. These SNP levels are in agreement with the SNP density level of 0.58% ($\sim 3.89 \times 10^6$ SNPs) as previously determined in genetic diversity study for the same parental lines (Aflitos *et al.*, 2014).

The SNP profiles in the genomic DNA molecules enabled us to assign COs according to the observed haplotype phase shifts. We first filtered ambiguous mappings, discarding discordant reads and molecules significantly overlapping with tomato genome repeats. Additionally, we removed recombinant molecules containing phase shifts with a skewed read coverage, that may have arisen from unknown repeat elements or copy number variation. Furthermore, we rejected multiple haplotype shifts, thereby avoiding cases where multiple putative COs or GCs

occurred in the same molecule. These filtering constraints rendered a total of 5126 recombinant molecules on 12 chromosomes. We also anticipated on the occurrence of possible non-crossover associated (NCO-GC) and CO-associated gene conversions (CO-GCs) as possible products of recombination events. Indeed, a previous study on targeted recombination analysis in tomato reported on a single discontinuous GC of 5–6 kb. This GC however, was observed in somatic tissue with artificially induced double stranded breaks (DSBs) by Cas9 (Filler Hayut *et al.*, 2017). In rice the average length of a gene conversion was reported to be 130 bp (Xu *et al.*, 2008). Considering that the observed average length of NCO-GCs and CO-GCs in *A. thaliana* ranges between 25–400 bp (Wijnker *et al.*, 2013), the additional filtering for tomato GCs left 3819 recombinant molecules representing candidate CO regions with haploblock sizes larger than 400 bp, a resolution (i.e. distance between markers flanking a CO) ranging from 2 bp to 213 kb, and spanning distance (i.e. between the first and last markers within a recombinant molecule)

Figure 1. (a) Schematic representation of crossovers and gene conversions detected from haplotype shifts. Each molecule (m) consists of linked reads displayed as arrowed blocks connected by a horizontal line indicating their linkage. The red and green markers indicate the parental origin of the allele. Molecule m_1 represents a recombinant molecule showing phase shift marking a crossover. The resolution (res) of the crossover is defined as the length between two consecutive phase shifting markers, while spanning distance is the distance between the first and the last marker in the molecule. Molecule m_2 contains a gene conversion with two supporting markers with a minimal and maximal tract length denoted by x and y , respectively. For GC detection the maximum tract length (x) was set to 400 bp. Molecule m_3 is non-recombinant, while in molecule m_4 the putative CO is discarded because its block length is shorter than 400 bp and displays only one phase shift. (b) Spanning distance versus crossover resolution with a CO represented either by a green or blue dot. Blue dots mark COs that were used to find correlations with sequence features. The dashed line represents the mean spanning distance of 55.9 kb.

(Figure 1a) from 1.9 kb to 297 kb (mean 55.9 kb, Figures 1b and Figure S4a), respectively. The resolution of each molecule is negatively correlated with the coverage of reads overlapping a SNP marker (Pearson correlation coefficient $r = -0.59$, P -value $< 2.2 \times 10^{-10}$) (Figure S4b), suggesting that increasing the sequence coverage would likely further improve the resolution.

A single homologous CO event results into two reciprocal recombinant molecules. When mapped against the reference genome, such a pair of overlapping recombinant molecules would display reciprocal haplotype profiles and point to the same phase shift position. However, currently we cannot precisely establish the actual total number of CO events from recombinant molecules. Sizes may differ between molecules because of random DNA breaks during library preparation and sequencing. Furthermore, molecules are randomly primed, and when sequenced, likely will differ in their read profiles. Also, recombinant molecules originating from multiple gametes and generated by multiple CO events, may still map to overlapping positions in the reference genome. In addition, we cannot identify the recombinant chromosome pairs that originate from a single gamete. These limitations prevented to link recombinant molecules with overlapping CO regions to a distinct number of CO events that generated them. Nonetheless, to determine the minimum number of CO, we screened 3819 CO regions, and assigned sets of recombinant molecules with overlapping CO regions to individual recombination events. This left a minimum of 3169 putative CO recombination events. We then manually checked phase shift blocks for inconsistencies in 50 randomly selected candidate CO regions. Taking advantage of the Integrative Genome Viewer (IGV) displaying barcode (BX) and molecule identifier (MI) annotation tags called by LONG RANGER, we found a false positive rate of 2%. Furthermore, the flanking sequences for all randomly selected CO regions showed an unambiguous BlastN hit to their corresponding position in the tomato reference genome. We therefore anticipated assigned COs resulting from ambiguous read mappings less likely. We also found a significant overlap between COs in pollen and in RILs (Demirci *et al.*, 2017) (P -value = 8.44×10^{-23} , Fisher exact test) using *bedtools fisher*. While our stringent filtering method greatly improved accurate detection, it likely decreased sensitivity though. Given the input gDNA, representing about 625 recombined F1 genome copies, and the previously reported average recombination rate in tomato of 1.8 per chromosome pair (Sherman and Stack, 1995), the theoretical number of COs in our pollen sample would be 13 500. The theoretical maximum number of COs, when compared to 3169 detected COs in our pollen sample, suggests a considerable number of COs were left undetected. However, considering that interspecies CO rates (i.e. between the homeologous chromosomes of tomato and

pimpinellifolium) can decrease to less than 20% compared to intraspecies recombination frequency (Canady *et al.*, 2006; Demirci *et al.*, 2017), the actual total number of COs in the F1 hybrid pollen presumably was lower. In the case of an interspecific hybrid, homeologous chromosomes will to some extent be different in terms of genomic (repeat differences), and cytogenetic (heterozygosity for smaller structural chromosome variants) differences. Previously, chromosome structural differences have been revealed between *S. lycopersicum* and *S. pimpinellifolium*, including mismatched kinetochores foldbacks and other irregularities (Anderson *et al.*, 2010a,b). Such differences between homeologous chromosomes lead to disturbances in the synapsis of the homeologues, and hence may dramatically decrease formation of CO events. Depending on the species in the tomato clade used, genetic lengths of individual chromosomes have been found to vary substantially (Bernachii and Tanksley, 1997). In addition, strong variation in recombination frequency has been shown dependent on the sex identity of the gametes. In plants, male and female meiosis differ considerably. Less recombination for male gametes was found in an F1 plant from a backcross to both the *S. lycopersicum* and *S. pennellii* parents (De Vicente and Tanksley, 1991). In contrast, Drouaud *et al.* (2007) found an elevated recombination frequency in male gametes from *Arabidopsis*, whereas Philips *et al.* (2015) reported on a higher recombination frequency for female gametes of barley and showed recombination rates are temperature dependent. Thus, caution should be taken when comparing recombination frequencies and genetic map lengths such as for species in the tomato clade and for plants in general. Although the average frequency of ~ 0.42 COs per recombined F1 pollen chromosome represents 23% of the interspecies CO frequency, it nevertheless falls within the range as previously determined for an F6 RIL population (Demirci *et al.*, 2017).

Variation in CO frequencies

The frequency of COs between haploid nuclei can directly be compared since all genome copies originated from one F1 plant, eliminating a possible bias in recombination that would be due to differences in genetic background. To determine distributions, we assigned COs to 50 kb bins ordered along each of the 12 tomato chromosomes. We made a distinction between euchromatin and heterochromatin positions as previously established (Sherman and Stack, 1995; Demirci *et al.*, 2017). As expected, COs were not uniformly distributed. In all chromosomes a higher frequency was clearly observed for euchromatin portions, while pericentromeric heterochromatin regions were mostly devoid of COs (Figure 2a,b). As previously suggested, the low number of COs in pericentromere domains probably occurred within small euchromatin islands harbouring actively transcribed genes (Peters *et al.*, 2009; The

Figure 2. (a) Distributions of crossovers in the chromosome arms of pollen gametes. Positions of centromeres for division of chromosomes into long and short arms and euchromatin and heterochromatin sections were taken from Demirci *et al.* (2017). (b) Distribution of CO in pollen chromosome 1 in 100 kb bins. A schematic representation for tomato chromosome 1 is shown above the graph with euchromatin (dark blue) and heterochromatin (light grey), and centromere (dark grey).

Tomato Genome Consortium, 2012). To further measure the propensities of the F1 pollen chromosomes toward COs, we compared sequences of 283 high-resolution CO regions (<1 kb) against the tomato bacterial artificial chromosome (BAC) fingerprint map. These high resolution COs do not share overlapping flanking markers and thus were likely generated by independent recombination events. The sequence comparison yielded 167 CO regions overlapping with tomato BACs anchored to the tomato EXPEN2000 genetic map. The order for 91 out of 95 genetic markers associated with the anchored BACs coincided with the mapping order of all high-resolution COs to the tomato reference genome, indicating it is unlikely that these COs were called from aberrantly mapped reads (Table S1). As expected, the frequency of COs in the heterochromatic regions is lower than in euchromatic regions (Figure 2a,b). Furthermore, we observed a higher CO frequency for euchromatin regions toward the distal chromosome ends, except for chromosome 2 which showed a higher frequency towards the end of the long arm. These CO profiles

in the F1 hybrid are in line with previously observed recombination profiles in tomato (Peters *et al.*, 2009, 2012, Aflitos *et al.*, 2014; Demirci *et al.*, 2017, 2018).

Correlation with primary sequence and gene features and hot and cold CO regions

To obtain a detailed recombination profile, we assessed the sequence divergence and distribution of a subset of 400 CO regions (Figure 1b). In order to retrieve a sufficient number of datapoints, we relaxed the resolution threshold to 1500 bp, which was 500 bp lower compared to the threshold for genomic sequence feature enrichment detection. These COs regions are not overlapping and thus were also likely generated by independent recombination events. When comparing CO regions to the annotated reference genome, a permutation test showed a significantly higher rate of COs (P -value = 1.0×10^{-6}) in gene promoter regions (Figure 3a). This is in line with earlier results, reporting on COs biased toward regions upstream of transcription start sites (TSS) in tomato (Demirci *et al.*, 2017),

Figure 3. (a) Frequency distribution of COs observed in genic features. The observed and expected CO frequency per class is represented by blue and orange bars respectively. **** $p < 0.0001$. (b) Enriched sequence motifs in CO regions as discovered with MEME.

rice (Wu *et al.*, 2003; Si *et al.*, 2015; Demirci *et al.*, 2018), maize (Rodgers-Melnick *et al.*, 2015), and *A. thaliana* (Wijnker *et al.*, 2013; Choi *et al.*, 2018). To check whether the enrichment in the 5'-UTR is an artefact of skewed SNP density causing the CO regions to extend from the promoter to the 5'-UTR, we focused on a subset COs, overlapping either a promoter or 5'-UTR and excluded COs spanning both regions. The additional permutation test on this subset confirmed the enrichment of COs in the promoter (z-score = 2.29, P -value = 0.0126) and 5'-UTR (z-score = 2.04, P -value = 0.0202) (Figure S5). Moreover, using the MEME suite (Bailey *et al.*, 2009), we identified significantly over-represented sequence motifs in 283 high resolution (below 1 kb) CO regions. The motifs were composed of poly-AG, poly-T and poly(AT) sequences, occurring in 282 ($E = 6.2 \times 10^{-24}$), 266 ($E = 2.1 \times 10^{-20}$) and 280 ($E = 9.0 \times 10^{-10}$) CO regions, respectively (Figure 3b). Poly-T and poly(AT) signatures are typically found at non-coding and promoter regions, and often at transcription

factor (TF) binding sites (O'Malley *et al.*, 2016; Cherenkov *et al.*, 2018) and have been found associated with cross-overs for example in *Arabidopsis* (Choi *et al.*, 2013; Wijnker *et al.*, 2013; Shilo *et al.*, 2015) and tomato (Demirci *et al.*, 2017, 2018). Previously, we also found a weak correlation with AG dinucleotides (Demirci *et al.*, 2017, 2018), further in line our observation of high CO incidence in gene promoter regions.

In addition, the observed distributions of recombinant sites along chromosomes suggested 'hot and cold regions'. Using CO density estimates, such regions with CO frequencies significantly deviating from the average were observed (Figure 4). We identified 23 hot regions in euchromatin portions except for chromosomes 3 and 6. Furthermore, using a *bedtools fisher* test a significant number (p -value = 4.77×10^{-05} , Fisher exact test) of 15 out of 91 cold regions with a minimum length of 100kb significantly overlapped with previously reported cold regions in F6 RILs (Demirci *et al.*, 2017), further suggesting that

Figure 4. Distribution of recombination sites in F1 tomato hybrid chromosomes. Heterochromatin, euchromatin, and centromeres are depicted as dark and light grey horizontal bars, and dark grey circles respectively. CO density graphs are superimposed on each chromosome. Crossover-dense (hot) and crossover-depleted (cold) regions in F1 hybrid pollen are shown as red and blue dots along each chromosome, respectively. Chromosome positions for NCO-GCs are indicated by pink triangles.

profiles in pollen gametes are representative for CO profiles observed in plants.

Gene conversion designation

Evidence has been presented for NCO-GC and CO-GCs in the animal and plant kingdom (Parniske *et al.*, 1997; Xu *et al.*, 2008; Duret and Galtier, 2009; Wijnker *et al.*, 2013; Trombetta *et al.*, 2016). When polymorphisms between alleles are involved, GCs in offspring alleles will be marked by SNPs, denoting the parental origin. We expected GCs also to occur during gametogenesis in our F1 tomato hybrid, though as far as we know a comprehensive genome wide analysis of GCs in interspecific tomato hybrids has not been presented until now. Both CO-GC and NCO-GC would consist of a small segment, containing a few SNPs marking a phase shift. Indeed, GCs observed in other plants have been reported in the 25–400 bp range (Xu *et al.*, 2008; Wijnker *et al.*, 2013). However, without knowing the recombinant pairs of chromosomes, our screening method currently can only detect NCO-GCs, occurring in small phase blocks less than 400 bp (Figure S6). After

selecting candidate GCs < 400 bp and subsequent manual validation of supporting linked reads, we indeed detected 44 gene conversions within tracts between 35–369 bp. These 44 NCO-GCs do not share overlapping regions and thus were likely generated by independent recombination events. We prioritized on accuracy rather than sensitivity, by screening for small domains containing exactly one GC and at least 2 SNPs whose identity could be traced to the homologous donor allele. It is likely that the full chromosome complement is subject to GC and we indeed found footprints in all chromosomes. However, our stringent filtering did not leave GCs for chromosomes 2 and 11. We found read mappings at 3 GC sites showing high quality SNPs (Phred-score >30) in addition to the expected polymorphisms that were detected between the parental homologous tomato and *pimpinellifolium* alleles. As these additional SNPs, occurring in a single read, could not be traced back to the parental alleles, we refer to them as ‘non-parental’ SNPs. The other linked reads generated from the same molecule flanking these non-parental SNPs mapped unambiguously to their homologous position.

Using BLAST, we found for example a read with non-parental SNPs at a GC in chromosome 1, consisting of a segment specific for the target allele and another segment mapping to a nearby paralogous region in a reversed orientation approximately 250 bp downstream of the conversion (Figures S7a and S7b). Considering that such clustered non-parental SNPs occurred in small segments identical to a parental paralogous allele, ectopic GC is the most parsimonious explanation. In addition, a separate set of 25 conversions that were reported as candidate GCs included insertions or deletions in a homopolymeric region. These indels, however, were not detected in the homologous or a paralogous allele. Currently, we cannot explain these indels by GC. We speculate they may have arisen as a result of illegitimate recombination such as non-homologous end-joining, or by polymerase slippage during replication, although sequencing errors of the homopolymeric region cannot be excluded.

DISCUSSION

Here we present an algorithm that faithfully detects phase shifts marking both meiotic crossovers and non-crossover associated allelic and ectopic gene conversions in haploid male gametes of a *S. lycopersicum* × *S. pimpinellifolium* hybrid, based on 10X Genomics linked read sequencing data. The algorithm identifies the parental origin of homozygous SNPs from linked reads of pollen gametes that have been mapped to the SL3.0 Heinz reference genome. The previously established high quality reference genomes of the *S. lycopersicum* cv. Heinz and *S. pimpinellifolium* parental lines (Aflitos *et al.*, 2014) facilitated stringent filtering of false positives resulting from base calling or read mapping errors, and allowed for accurate CO and GC calls from phase shifts as presented here. Although, we couldn't establish the precise number of COs, because pairs of overlapping recombinant molecules do not have the same read profile and size, obscuring the identity of recombinant pairs in linked read data, we related non-overlapping recombinant molecules to a minimum number of CO events that generated them. Our analyses showed CO enrichment in promoter regions and the occurrence of specific sequence motifs consistent with previous results (Demirci *et al.*, 2017, 2018). In addition, we now also show an elevated CO frequency in the 5'-UTR regions. Homologous meiotic recombination has been shown to occur around the transcription start sites and nucleosome-depleted regions, suggesting that open chromatin is accessible to diverse cellular machineries (Pan *et al.*, 2011; Choi *et al.*, 2013; Wijnker *et al.*, 2013; Shilo *et al.*, 2015; Demirci *et al.*, 2017, 2018). A diversity analysis of 529 *Arabidopsis* TF-binding motifs showed that 25% have at least 4 consecutive A/T stretches (O'Malley *et al.*, 2016). These A/T rich motifs have been found overrepresented for example in TF-binding sites related to auxin response in *Arabidopsis*

(Cherenkov *et al.*, 2018). A relationship between transcribed genes and the proximity to recombination hotspots was reported for yeast (Mancera *et al.*, 2008). Furthermore, AT-rich domains appear to disfavour nucleosome occupancy and recently have been shown to be involved in the formation of DSB and hotspots of recombination in *Arabidopsis* (Choi *et al.*, 2018). The enhanced recombination in both promoter and 5'-UTR regions might thus be explained by the local structural conformation of the DNA that is accessible for replication, recombination, and DNA transcription complexes as previously suggested (Gottipati and Helleday, 2009; Demirci *et al.*, 2017, 2018). The CO preference at promoter regions and CO hot and cold spots significantly overlapping with the regions of elevated and repressed CO in F6 RIL plants, further suggests that we faithfully detected COs and GCs in pollen gametes. It is possible that we have missed COs, especially in regions devoid of SNPs or with low coverage. However, since the observed recombination frequency in haploid spores was in the same range as previously observed for tomato hybrids (Demirci *et al.*, 2017, 2018), we speculate that the number of missed COs is relatively low. Nevertheless, approximately 5×10^6 heterozygous SNPs and 4.7×10^5 indels have not been taken into consideration as potential CO markers yet, and may serve to further improve the resolution and sensitivity of CO and GC detection from linked read data. Additional adjustments, such as increased read coverage, and allowing for multiple phase shifts per molecule, also might improve the sensitivity. Although, we currently do not consider recombination events from multiple phase shifts, molecules with complex phase patterns in ranges between 30–100 kb were detected. We do not rule out complex GC in tomato, rather these observations hint at a tomato recombination machinery that is more complex than currently anticipated. Indeed, high-resolution mapping of meiotic crossovers and non-crossovers for example in yeast showed conversion tracts exhibiting complex patterns of genotype change, probably resulting from repair of multiple patches of heteroduplex, or alternatively, mismatch repair switching between conversion and restoration (Mancera *et al.*, 2008; Leseque *et al.*, 2013). Furthermore, the involvement of tomato transposable elements as drivers of meiotic recombination cannot be ruled out, since we observed putative GC in recombinant molecules overlapping with repeats and transposon copies. Previously, meiotic recombination and GC induced by the *Mutator* (*Mu*) transposon in maize, resulted in deletions affecting gene function of the *knotted 1* gene (Mathern and Hake, 1997). A study on the trans-acting regulatory *MuDR* transposon revealed increasing GC and intragenic meiotic recombination in the vicinity of a nonautonomous *Mu* transposon (Yandeau-Nelson *et al.*, 2005). Furthermore, maize centromeres consisting of an abundant class *Ty3/Gypsy* like transposable elements, known as Centomeric

Retroelements, apparently exhibited widespread GC (Shi *et al.*, 2010). We nevertheless currently prioritize on accuracy rather than sensitivity, excluding molecules overlapping with repeat and transposon copies to avoid aberrant recombination events called from misaligned recombinant molecules. This limitation could be overcome by lowering the molecule per GEM ratio and increasing the average length per molecule, sustaining faithful molecule mappings at a higher SNP levels, which then could shed more light on a possible role of transposons in meiotic CO recombination and CO associated and NCO associated GC in tomato.

Recombination profiling in plants has seen many techniques and approaches (Lambing and Heckmann, 2018). Among them are profiling strategies for male gametophytes which have relied on amplification of randomly amplified polymorphic DNA and single sequence repeat markers (Chen *et al.*, 2008), tetrad analysis of *Arabidopsis* *qrt* mutants (Copenhaver *et al.*, 2000; Wijnker *et al.*, 2013), whole genome amplification (WGA) of DNA of male spores (Aziz and Sauve, 2008) in combination with FACS sorting and KASP genotyping (Dreissig *et al.*, 2015), and recently also linked read sequencing of *Arabidopsis* *Col* × *Ler* F1 gametes (Sun *et al.*, 2019) and gametes from mouse and stickleback (Dreau *et al.*, 2019). While linked-read sequencing of pollen gametes and sperm cells enabled high-throughput profiling of COs, NCO related recombination events were not reported (Sun *et al.*, 2019; Dreau *et al.*, 2019). The genome coverages in these linked-read based profiling studies were considerably higher (~170×) compared to our genome coverage of approximately 100×. We could not retrieve information on the map-based coverages in the aforementioned studies, which prevented further comparison to our map-based coverage of 68×.

The strategy described in this study has both fundamental and applied research implications. Its high throughput enables profiling of pooled recombination events, without the need to screen an offspring population. Our screening requires a pollen sample from a single F1 offspring plant, which makes the technology applicable for screening many parental breeding line combinations. Such screenings could for example provide guidance for selection of compatible donor and recipient genomes to overcome linkage drag problems. Since our method requires a single generation, it also becomes practical to profile recombination in crops with relatively long generation periods. Crossover recombination can now be assessed relatively fast and cheap with unsurpassed precision down to a resolution of 2 bp as we have shown here. It thus becomes possible to assess the effects of genetic background, genome structure, and environmental conditions on recombination in greater detail and at a higher response level. This technology can thus boost breeding of advanced crops aimed at maintaining food security and safety, which is urgently

needed in the face of the socio-economic and environmental challenges that traditional plant breeding currently faces (Nature Plants editorial, 2018; Nature editorial, 2018). Furthermore, recombination profiles from genetically diverse panels that are tested under variable environmental conditions can be used in a machine learning approach to improve our current CO prediction models (Demirci *et al.*, 2018), providing further insight into gross similarities between species and species-specific aspects of recombination.

The current understanding of the recombination machinery in tomato is still incomplete as suggested by the complex patterns of exchanges that we detected. For example, besides 41 allelic GCs observed in both euchromatin and heterochromatin portions of the chromosomes, we detected 2 ectopic GCs in chromosome 1 and one in chromosome 3 in a reversed configuration relative to a nearby paralogous allele. We currently cannot explain the reversed configuration, but speculate that small inversions, as shown in mouse and *Solanum* hybrids, may cause local inversion loops in synapsed chromosomes (Borodin *et al.*, 1991; Sherman and Stack, 1995; Anderson *et al.*, 2010a,b). A DSB in such inversion loops may perhaps be resolved into a gene conversion with an inverted orientation relative to the donor allele. Interestingly, a number of studies report on intrachromosomal DSB repairs in plants which, depending on a direct-repeat or inverted-repeat configuration of the involved locus, were thought to have been processed via the single strand annealing (SSA) or synthesis-dependent strand-annealing (SDSA) pathway, resulting either in a deletion or a gene conversion respectively (reviewed in Puchta, 2005; Morrical, 2015). Furthermore, we observed 25 conversion tracts that cannot be explained by homologous CO or GC only. Careful breakpoint analysis showed that these recombinants contain indels that were not observed in the parental homologous or paralogous alleles. Our current analyses remain indecisive to the origin of these additional indels. These polymorphisms could have arisen for example by polymerase slippage during sequencing. Alternatively, these polymorphisms may have been introduced via alternative repair pathways such as non-homologous end joining (NHEJ) (Puchta, 2005). Indeed, micro-homology studies in human, have pointed at NHEJ and template-switching mechanisms generating small additional indels at repaired breaks (Kidd *et al.*, 2010; Abyzov *et al.*, 2015). Interestingly, interrupted GC was shown to generate truncated *Mu* elements in maize. The interruption was proposed to result from hybridizing direct repeats, upon which the gap resolution would lead to a deletion (Mathern and Hake, 1997). In our data we occasionally detected GCs associated with small deletions in recombinant molecules overlapping transposon related repeats. Whether transposon related repeats around these putative recombination events in tomato mediated

deletions at repaired breaks, currently remains speculative though. Furthermore, NHEJ has been reported in yeast, mammals, and *Drosophila* to repair double stranded breaks by direct re-joining without extensive processing, frequently introducing unequal sequence exchanges such as indels (reviewed in Caridi *et al.*, 2017). The recruitment of the HR or NHEJ pathway seems to be dependent on the chromatin compaction as has been observed in human where DSB in heterochromatin appears more prone to NHEJ than HR mediated repair (Caridi *et al.*, 2017; Nisa *et al.*, 2019). NHEJ might partly explain the conversions that we observed in the pericentromeric heterochromatin of F1 tomato hybrid chromosomes. Moreover, combinations of HR and NHEJ mediated repairs have been reported to contribute to a complex mosaic of exchanges at repaired breaks (Puchta, 2005). Recently, it was demonstrated that DSB in repetitive 45S rDNA loci from *Arabidopsis*, during meiosis are shielded from HR and preferentially repaired via NHEJ (Sims *et al.*, 2019). Whether the occurrence of such additional indel markers can be attributed to the aforementioned NHEJ mediated repair mechanism during meiotic recombination in tomato, currently remains speculative and will be subject to further study.

EXPERIMENTAL PROCEDURES

Selection of tomato accessions

An F1 plant was produced from an interspecific cross between *S. lycopersicum* cv. Heinz 1706 and *S. pimpinellifolium* CGN14498, which was used as the male and female parent respectively as described in Aflitos *et al.* 2014. The F1 plant was grown to flowering in a greenhouse under normal day night light (16 h/8 h) conditions at 24°C.

High molecular weight DNA isolation from pollen

Mature pollen was released from flowers using a handheld milk frother device and collected in a 1.5 ml tube. Pollen were then stored at 4°C at a relative humidity of 32% or immediately germinated after harvesting according to the protocol described by Lu *et al.* 2015 with modifications. Approximately 2–10 mg pollen were covered with a small gauze and positioned in a small petri dish containing saturated Na_2HPO_4 for 4–8 h at 25°C without bringing the pollen into direct contact with the solution. The hydrated pollen were then incubated in 2.5 ml germination medium (20 mM MES, 3 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 1 mM KCl, 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mM Boric acid (H_3BO_3), 24% (w/v) PEG4000, 2.5% (w/v) sucrose at pH 6.0 for 1.5 h at 25°C, shaking at 90 rpm in the dark. Germination was monitored by assessing small samples with a light microscope at 20× magnification and was terminated when pollen tubes reached approximately 2× the diameter of the pollen grain. Germinated pollen were isolated from the medium with a 20 µm cell strainer (pluriStrainer, pluriSelect Life Sciences, Leipzig, Germany), collecting the flow through in a 50 ml tube by washing with 1 ml nuclei isolation buffer (0.3 M sucrose, 10 mM NaCl, 10 mM 2-(N-morpholine)-ethanesulphonic acid, 5 mM ethylenediamine-tetraacetic acid, 0.2 mM spermine, 0.5 mM spermidine, 0.2 mM phenylmethylsulphonyl fluoride, 5 mM dithiothreitol, and 0.2% (v/v) Triton-X-100, adjusted to pH 7.4 with 2 M

NaOH). Nuclei were subsequently isolated from pollen according to Gao *et al.* (2013) with modifications. Subsequently, nuclei were collected by placing the 20 µm pluriStrainer with a connector ring on top of a 10 µm pluriStrainer and then put on top of a clean 50 ml tube. The 20 µm cell strainer was used to isolate the released nuclei and the non-germinated pollen from the germinated pollen, while the 10 µm cell strainer was used to separate the released nuclei from the non-germinated pollen. Next 1 ml nuclei isolation buffer was added to the pollen sample and nuclei were subsequently released by gentle homogenisation of the pollen tubes using a pestle for cell strainer (Sigma-Aldrich, Zwijndrecht, The Netherlands) at 60–100 rpm for 1 min. Nuclei were rinsed from the 10 µm pluriStrainer and collected in a 50 ml tube and centrifuged three times at 500 g for 5 min to pellet the nuclei. The nuclei were then embedded in 2% liquid low melting point agar kept at 45°C. The pellet was suspended in 10 µl nuclei isolation buffer, raising the temperature gently to 45°C before adding 20 µl of 2% Low Melting Point (LMP) agarose at 45°C and casting the solution into a mould. Plugs containing the nuclei can either be stored in TE buffer at 4°C, or directly be used for high molecular weight DNA isolation from the plugs according to the BioNano Genomics extraction protocol 'IrysPrep Plug Lysis Long DNA isolation' (30026D) as described by the manufacturer. The DNA concentration and molecule length was determined with a Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and a DNA Fragment Analyzer (Agilent, Santa Clara, CA, USA).

10X Genomics library preparation and sequencing

The 10X Genomics libraries were constructed with the Chromium™ Genome Reagent Kits v2 (10X Genomics®) according to the Chromium™ Genome v2 Protocol (CG00043) as described by the manufacturer. Sequencing was carried out using Illumina HiSeq 2500 with 2 × 150 bp paired-end reads to a genome coverage of approximately 100X. The LONG RANGER pipeline from 10X Genomics was used to process the sequencing output and align the reads to the tomato reference genome (The Tomato Genome Consortium, 2012). The LOUPE genome browser summary view from 10X Genomics was used to retrieve the metrics on input DNA, sequencing, molecule length, coverage and gel beads in emulsion (GEM) performance. The number of F1 hybrid genome copies that were assessed was calculated using Lander-Waterman statistics (Lander and Waterman, 1988).

Sequence alignment and data processing

Illumina PE sequences from RIL parent *S. pimpinellifolium* CGN 14498 and from *S. lycopersicum* cv. Heinz from the 150 Tomato Genome Project (Aflitos *et al.*, 2014; Demirci *et al.*, 2017) were aligned to the *S. lycopersicum* cv. Heinz reference genome v3.0 using the read mapper tool from CLC Genomics workbench v11.0.1 (<https://www.qiagenbioinformatics.com/>). Reads were subsequently realigned to adjust for local mapping inconsistencies near insertions and deletions using the local read mapping alignment tool from CLC and the indel realignment GATK toolkit v.4. Homozygous and heterozygous single nucleotide variants (SNPs and indels) were then called using GATK UNIFIEDGENOTYPING and the fixed ploidy level variant detection algorithm from CLC separately. For GATK we used recommended parameter settings as described (<https://software.broadinstitute.org/gatk/documentation/article.php?xml:id=3225>), whereas for the CLC fixed ploidy detection algorithm we used the following settings: Ploidy = 2; Required variant probability (%) = 80.0; Ignore positions with coverage above = 100 000; Ignore broken pairs = Yes; Minimum coverage = 8; Minimum count = 2; Minimum frequency (%) = 20.0;

Neighborhood radius = 5; Minimum central quality = 20; Minimum neighborhood quality = 15; Relative read direction filter = Yes; Significance (%) = 1.0. Homozygous *S. pimpinellifolium* SNPs predicted by both GATK and CLC were then intersected and selected as segregating markers, excluding all positions found to be polymorphic between the tomato parent and the reference genome. Chromium barcoded sequences from the pollen sample were processed and aligned to the *S. lycopersicum* cv. Heinz v3.0 reference genome (https://solgenomics.net/organism/Solanum_lycopersicum/genome) using the LONG RANGER pipeline with default parameter settings. The output was processed by SAMTOOLS v1.3 *calmd* to generate the MD tags and then filtered per chromosome. Paired end (PE) reads without barcodes and molecule IDs, having a mapping quality below 60, showing an abnormal template length larger than 2 kb, or discordantly aligned to multiple chromosomes, were removed. The resulting alignment file was intersected with the list of *S. pimpinellifolium* homozygous SNPs to retrieve reads relevant to the phase shift detection. Reads having identical barcode (BX), molecule identifier (MI) and alignment start position were marked as clonal reads and discarded. Both CIGAR and MD tags of non-clonal reads were then parsed to assign the parental origin of the polymorphism that matched a segregating marker. The haplotype for each linked read molecule was determined using the pipeline illustrated in Figure S1. Briefly, putative haploblock shifts were assigned to a linked read molecule if two adjacent SNP blocks, consisting of at least three uninterrupted and contiguous SNP markers, were assigned to different parents, i.e. 'aaabbb' or 'bbbaaa'. CO recombination sites were then called taking the midpoint position between the two SNP markers flanking the phase shift. Molecules with candidate COs were subsequently filtered against tomato repeats (ITAG3.2_Repeat Modeler_repeats), chloroplast insertions in the tomato nuclear genome, and tomato retrotransposons (tomato_retrotransposons_PRI_v2) retrieved from ftp://ftp.solgenomics.net/genomes/Solanum_lycopersicum/repeats. To avoid aberrant haplotype shifts due to misalignment of tandem repeats aligned molecules overlapping at least 10% in length with repeats or candidate CO regions having a read coverage of flanking markers higher than 95% of all marker coverages across the genome were discarded (Figure S2). To focus on the unambiguous occurrences of GC, molecules with multiple haplotype shifts were excluded. A block of at least two SNPs with the same parental origin flanked by SNPs from the other parent were identified as gene conversion, i.e. 'aaabbaaa' or 'bbbaabbb'. Molecules with phased regions flanked by two phase shifts were selected as candidate gene conversions (GCs). Candidate GCs were retained if (i) they were at most 400 bp long, supported by SNPs having a high base quality score (Phred-score >30) matching with either parental allele, (ii) the supporting reads were not soft/hard-clipped, (iii) and did not overlap parental indel markers (to avoid false positives from aberrant indel marker calls). Remaining GCs containing indels that were not present in either homologous parental allele, but with supporting SNPs located within 3 bp distance of the indel, or GCs containing indels within homopolymeric regions of at least 4 bp were reported separately as they may have arisen from non-homologous directed repair mechanisms. Read alignments were visualized in the Integrative Genome Browser (IGV) (Robinson *et al.*, 2011) and used to manually examine the linked read support for randomly selected COs and GCs, including a check on consistent BX and MI identifiers, and a verification of the SNP identity with a Phred score 30 or higher matching to either parental allele.

Sequence motif discovery and gene feature distribution

Sequences of 1kb, flanking both sides of the midpoint of high resolution (<1 kb) CO regions ($n = 283$) were selected for motif discovery with the MEME suite using default settings (Bailey *et al.*, 2009). Only overrepresented motifs at least 6 bp long, occurring in at least 50 CO regions, were reported. To find possibly enriched gene ontology terms, motifs with significant E -value < 0.05 were submitted to GOMo (Buske *et al.*, 2010).

Identification of hot and cold regions (CO-dense and CO-poor regions)

Using the R-package function 'density', we estimated the CO density using a Gaussian kernel (bandwidth = 20 000 nt) for every 1000 nt. Euchromatic and heterochromatic regions were delimited as previously described (Demirci *et al.*, 2017). We then computed the p -value for local maxima and minima detected by the R function 'turnpoints' based on density estimates in random recombination region sets generated by shuffling the observed euchromatic crossovers in each chromosome 10 000 times. Significant p -values of density peaks or hot regions were adjusted for multiple testing using a Bonferroni correction with a 0.05 cut-off threshold. For density pits or cold regions, we apply the P -value cut-off of 0.05 without Bonferroni correction and merged consecutive pits that are at most 10 kb apart. Only cold regions covering at least 100 kb were reported.

Genetic marker order

Sequences from linked reads containing haplotype shifts, marking a potential crossover recombination, were compared against marker sequences from the Tomato-EXPEN-2000.v1 genetic map (<https://solgenomics.net>) using BLASTN and then filtered using an identity threshold of 98%. Positional marker orders between the linked read genetic map and the tomato genetic maps were compared to assess marker collinearity.

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AUTHOR CONTRIBUTIONS

Roven Rommel Fuentes and Sander A. Peters conceived and designed the analysis. Tamara Hesselink; Elio Schijlen, Ronald Nieuwenhuis, and Willem van Doijeweert collected the data. Roven Rommel Fuentes, Aalt D.J. van Dijk, Dick de Ridder, and Sander A. Peters contributed data or analysis tools. Roven Rommel Fuentes, Linda Bakker, Ronald Nieuwenhuis, Sander A. Peters performed the analysis. Roven Rommel Fuentes, Sara Diaz Trivino, Jorn R. de Haan, Gabino Sanchez Perez, Xinyue Zhang, Paul Fransz, Hans de Jong, Aalt-Jan van Dijk, Dick de Ridder, and Sander A. Peters wrote the paper.

CONFLICT OF INTEREST

The authors declare not to have conflicts of interests concerning this publication.

DATA AVAILABILITY STATEMENT

All the data used in this study are available from the corresponding author upon request.

SUPPORTING INFORMATION

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

Figure S1. Schematic representation of the crossover and gene conversion discovery pipeline.

Figure S2. Distribution of marker coverage and counts.

Figure S3. Variant classes of *S. pimpinellifolium* compared to *S. lycopersicum* parental genomes.

Figure S4a. Spanning distance versus crossover resolution with CO represented by a green dot.

Figure S4b. Correlation of molecule coverage and resolution.

Figure S5. Enrichment of COs in promoter and 5'-UTR regions.

Figure S6. Schematic representation of recombinant chromosomes with COs and GCs.

Figure S7a. Possible configurations involved in an ectopic gene conversion in chromosome 1.

Figure S7b. Sequence alignment of a linked read to repetitive alleles marking an ectopic GC in chromosome 1.

Table S1. Comparison of positional orders between crossovers and TOMATO-EXPEN 2000 genetic map markers.

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