1. דף שער

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דו"ח שנתי לשנת 2020 ומסכם

מחקר: **<u>מיפוי גנטי בעזרת עריכה גנטית</u>**

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2. תקציר

<u>הבעיה:</u> שונות גנטית מהבר נחשבת כמקור החשוב ביותר לטובת העלאת יבולי גידולים חקלאיים תחת סביבות עויינות המאפיינת את החקלאות בתקופה זו של שינויי אקלים. עם זאת, צוואר הבקבוק המשמעותי באיתור וניצול אותם אללים המשפיעים על התכונות השונות הינו חוסר האקראיות בשחלופים המתקיימים בין הגנומים השונים (בר ותרבות), כאשר איזורים צנטרומרים, לדוגמא, מאופיינים בתדירות רקומבינציה נמוכה באופן משמעותי. לשם כך נחוצות שיטות חדשות שיאפשרו הכוונה והעלאת תדירות של אירועי הרקומבינציה באופן שישלב זאת בצורה מיטבית עם תהליך הטיפוח.

<u>שיטות העבודה:</u> תכנון של מולקולות רנא המכווינות יצירת שברים דו-גדיליים והעברה שלהם לעוברים הטרוזיגוטיים על מנת לעודד רקומבינציה הומולוגית במיטוזה. עוברים אלו נבחנים בשלב ראשון של מחקר לתדירויות רקומבינציה ובשלב מאוחר יותר מתפתחים לצמחונים רקומביננטים שמהווים בסיס למיפוי של התכונה. השיטות כוללות 1) תכנון בניית קונסטרקטים בינריים והעברה שלהם לתאים עובריים דרך אגרובקטריום ; 2) תכנון ושימוש בקומפלקסי רנא-חלבון (CAS9 protein-Ribonucleotide gRNA) לירי חלקיקים על תאים מיטוטיים ; 3) הפקות דנא ובחינת תדירויות רקומבינציה הריצוף דור-חדש ובעזרת ddPCR ; 4) רגנרציה וגידול צמחים רקומביננטיים לאיפיון פנוטיפי.

<u>תוצאות עיקריות:</u> הושגו שלושת המטרות הראשונות, קרי תוכננו ויוצרו קומפלקסי CAS/gRNA לשני אתרים בגנום ואלו הראו פעילות חיתוך ספציפית על פלסמידים שמכילים את גן המטרה. ביוליסטיקה לתאים מיטוטיים של גן מדווח הראתה חדירות גבוהה במיוחד והושג פרוטוקול עבודה ליצירת קאלוסים ורגנרציה של צמחי שעורה מזרעים בוגרים. בנוסף, תהליך הביוליסטיקה של קומפלקסי CAS9/gRNA לשני גני מטרה נעשה על תאים מיטוטיים של הטרוזיגוטיים מתאימים בשני הלוקי, דנא הופק ונבחן על ידי digital droplet PCR ו Next generation sequencing לאיפיון וחישוב רקומבינציה בתאים המיטוטיים.

<u>מסקנות והמלצות לגבי יישום התוצאות:</u> על פי סיכום נתוני ה ddPCR ניתן להסיק כי אכן ניתן להעלאות רקומבינציה בין כרומוזומים הומולוגיים על ידי הפעלת שברים דו-גדיליים שמכוונים עם קומפלקס של CAS9 וgRNA. העלאת הרקומבינציה היא משמעותית לעומת הביקורת בלוקוס Heat3.1 ולא נצפתה ב לוקוס Dry2.2. הסיבה לחוסר יכולת ליצור רקומבינציה מיטוטית באחרון היא כנראה אינוורסיה כרומוזומלית בין האללים השונים כפי שדווח רק לאחרונה ואומת על ידינו. על מנת להמשיך ולבצע RECAS9ב לוקוס זה החלנו בהכלאות ברקע גנטי מתאים (ללא אינוורסיה כמו אלל הבר אך גם ללא השפעה על פנוטיפ).

השוואה בין תדירויות רקומבינציה המופעלת דרך CRISPR/CAS9 בתאי קאלוס מראה כי החדירות של התהליך גבוהה יותר בהעברה של ווקטור בינרי על ידי אגרובקטריום לעומת ביטוי חולף של הקומפלקס בירי חלקיקי זהב. ניסיונות לשפר חדירות על ידי ננו-חלקיקים אחרים לא עלו יפה. תוצאות מחקר זה מראות שניתן להכווין ולשפר רקומבינציה בתאים מיטוטיים על ידי עריכה גנטית ושניתן לשפר בעזרת RECAS9 תהליך מיפוי תכונות פשוטות ומורכבות. עם זאת, כרגע תהליך זה יעיל יותר בעזרת אגרובקטריום ומשמעותו שנוצרים צמחים טרנסגניים בתהליך. למרות שניתן להיפטר מהטרנסגן בהכלאות נראה כי ישנו פונטציאל בשיפור התהליך של RNP וזאת על ידי שיפור החדירות של הקומפלקס רנא-חלבון ושיפור תדירות ההתמרה לצמחונים. לחלופין, אוטומציה של התהליך ושימוש במספר רב יותר של עוברים הטרוזיגוטיים יובילו לשיפור סיכויי הצלחה.

3.1 INTRODUCTION AND BACKGROUND

Functional genomics using naturally-occurring alleles and genetic engineering often are regarded as an oxymoron, mainly due to a long history of failures in using quantitative trait locus (QTL) mapping and gene knock-out approaches for assigning function to gene variants. It is only high-resolution mapping of traits that allows delimitation of causal variation for complex traits to a single gene level (Frary et al., 2000)(Fridman et al., 2004). Bridging functional genomics and genetic engineering is key to unraveling mechanisms underlying complex traits, e.g. constructing a gene dosage series, or knock out (KO) of an identified gene (Liu et al., 2003)(Zanor et al., 2009), respectively. Nevertheless, finding rare gene alleles in gene pools still requires efficient and uniform recombination to allow linking of chimeric alleles to phenotype. In barley, as in other cereal crops, the low-recombining pericentromeric region of the genome contains roughly a quarter of the genes of the species (Baker et al., 2014). Recent studies have shown that Cas9 could assist in directing recombination in meiotic, but also in mitotic, cells. (Sadhu et al., 2016) transformed heterozygous *Saccharomyces cerevisiae* strains with series of sgRNA to generate a panel with recombination events spaced along the chromosome arm in the vicinity of a QTL for manganese sensitivity. Using this approach, fine-mapping the sensitivity to a single polymorphism in the transporter Pmr was accomplished. Nevertheless, this approach required the selection of cells undergoing loss-of-heterozygosity (LOH) using a GFP marker at the telomeric ends of the chromosome under study (since mitotic recombination leads to LOH). Similar Cas9-mediated recombination in plant mitotic cells, although not for trait mapping, also required stable transformation using *Agrobacterium*(Filler Hayut et al., 2017). Crossing the two transgenic lines, one with Cas9 (under a 35S promoter) and the other with gRNA (under a U6-26 promoter) targeting DSB induction between two alleles carrying different mutations in the PHYTOENE SYNTHASE gene (PSY1; *yellow flesh e3756* and *bicolorcc383*) resulted in yellow fruits with wild type red sectors. These results imply that Cas9 could achieve mitotic recombination, which could significantly enable trait mapping, but achieving this goal in a transgene-free manner and with QTL for complex and agriculturally-relevant traits remains challenging.

Agrobacterium-mediated delivery (Herrera-Estrella, Depicker et al. 1983) is the most commonly used tool for gene delivery into plants with limitations of efficient delivery

to only a narrow range of plant species, inability to perform DNA-free editing, unsuitability for high-throughput applications, and unavoidable DNA integration into the plant host genome (Baltes, Gil-Humanes et al. 2017). While some research is undertaken to prevent T-DNA integration with Agrobacterium transformation (Stoddard, Baltes et al. 2016), however the practice is still not developed enough for routine use in many plant species. The one other commonly used tool for plant transformation is biolistic particle delivery (also called gene gun) (Klein, Wolf et al. 1987), which can deliver biomolecules into a wider range of plant species but faces limitations of low-level and sporadic expression, plant tissue damage when high bombardment pressures are used (Altpeter, Springer et al. 2016), and the requirement of using substantial amount of DNA to achieve desired delivery efficiency. Notably, RNP-based biolistic delivery remains low-efficiency, presumably due to the nuclease loss of activity during dehydration onto the gold biolistic particle.

Our research project aims at utilizing Cas9 mediated recombination to fine-map QTL for different traits. Our starting plant material to zoom-in from chromosomal introgressions to sub-telomeric regions in the Halle Exotic Barley resource (Maurer et al., 2015). This multi-parental mapping population was designed to investigate regulation of different agronomic traits including flowering time. The population was developed by crossing 25 exotic donors of wild barley *Hordeum vulgare* ssp. *spontaneum* with the spring barley elite cultivar BARKE (H. *vulgare*) . After the initial cross, the plants were backcrossed to create BC1 hybrids with the common recurrent Barke cultivar, and then self-fertilized to generate 25 BC1S3 populations of app. 60 each, altogether 1,420 lines. This population was genotyped with 9000 iSelect SNP markers to determine genomic composition of cultivated and wild alleles. Several field experiments were conducted in several environments to identify wild alleles regulating different traits to identify, for example, drought-conditioned QTL such as the Hs*Dry2.2* (Merchuk-Ovnat et al., 2018). In this project, we followed a candidate QTL for heat tolerance Hs*Heat3.1* based on off-season growth of the population during 2016 (Khaled Bishara thesis, Bar-Ilan University), and the HsDry2.2 that we identified and published earlier (Merchuk-Ovnat et al., 2018).

Objectives of this project (updated and approved during 2020):

1. Generate the plant material for directing recombination in heterozygous plants by genome editing, i.e. RECAS9
2. Design and synthesize CAS9-gRNA ribonucleotide protein (RNP) complexes and test these in vitro
3. Deliver CAS9/gRNA to barley calli (mitotic cells) and characterize the recombination rate
4. Regenerate barley mitotic cells treated by RECAS9 to deirect recombination in two loci (Heat3.1 and Dry2.2) , and identify recombinants
5. Define the functional DNA variation underlie phenotypic variation in two loci

3.2 Materials and Methods

3.2.1 Plant material and genotyping

The HEB-25-049 plant material used in this experiment is originated from the barley NAM population HEB-25 (Maurer et al. 2015). This line with residual heterozygosity was further selfed to generate BC1S5 segregating seeds and plants for phenotype and RNP delivery experiments. A genotyping assay for the *Heat3.1* locus was developed using TaqMan®SNP assay. Each probe was designed to attach to a different nucleotide at in position 66,850,140 bp on chromosome 3H (A/T). The wild barley *Hordeum spontaneum* (Heat3.1^{Hs}) probe was labeled with Yakima yellow dye and the cultivated *H. vulgare* (Heat3.1^{Hv}) probe with FAM dye. We made 40X stock by combining 36µM and 8 µM of primer and probe, respectively, in a total volume of 100µl. We then used primers PM18.0020 and PM18.0021 (Supplementary Table 1) to amplify the desired region. Reaction mix for genotyping included: 10 ngr genomic DNA, 0.25 µl from the 40X stock in a 10 µl TaqMan®SNP assay mix. We made quantitative polymerase chain reaction (qPCR) steps as follows: Pre-PCR read 60°C, 30 sec; holding stage 95°C, 10min; 55 cycle of 95°C, 15sec; 60°C, 1min and finally a post-PCR 60°C, 30sec. Each assay included controls of plasmid or genomic DNA of known genotype (Heat3.1$^{Hs/Hs}$; Heat3.1$^{Hv/Hv}$; Heat3.1$^{Hs/Hv}$).

3.3.2 Field and circadian clock phenotyping

The field experiment was conducted in Agriculture Research Organization – Volcani center (ARO) in Rishon Lezion (E34º49', N31º59') in a nethouse during growing season 2017-2018. The nethouse is covered with a 50 mm MESH . Plants were sown into trays 171-hole planting trays containing soil mix (RAM 11), covered with

Saran wrap and incubated for 48 hr at 4ºC to allow uniform germination, and moved to nethouse for further germination until transplanting (transplanting date January, 2017). Net house was divided into two parts: Eastern part that included fan was uncovered, while western part was covered with polyethylene, and three electric fan heaters (GL3-3000W) were installed to have heat stress effect. For planting we used two 6 x 0.3 m troughs (Mapal Horticulture Trough System, Merom Golan, Israel) filled with lower layer of 70% tuff medium and covered with Odem soil type (Toof Merom, Golan, Israel). Two-leaf stage seedlings were transplanted to both sides of each trough with 10 cm distance between plants. For each trough one irrigation dripping pipe (30cm between each drip points) was installed. In order to ensure no drought or limited nutrient stress developed, plants where water irrigated 3 times a day combined with NPK fertilizer. We scored the genotype of all individual plants using a TaqMan®SNP assay (see above) and plant phenotyping was conducted as described previously (Merchuk-Ovnat et al., 2018).

In the circadian clock experiments, 24-36 BC1S5 plants were sown in 6-pack plastic trays (Tivan Biotech #1206 and #1020) and moved to 4ºC for at least 24 for unified germination. The SensyPAM room is thermally controlled thus allowed us to conduct experiments in high temperatures (HT, 34ºC) and optimal temperatures (OT, 22ºC) under continuous light. We made experiments in two environments to calculate the plasticity of the clock or the delta between values at HT and OT(Bdolach et al., 2019). The experiments started under OT conditions for 3 days in SensyPAM. We then returned the plants for three days under long days (14 hr of light) for entrainment in the growth room, before continuation to HT experiment for three more days. The F measurements of leaf areas were used to calculate photosynthesis parameters including NPQlss $\frac{fm-fmlss}{fmlss}$following (Bdolach et al., 2019). NPQlss was then used to calculate the free running period (FRP), amplitude (AMP), and relative amplitude error (RAE). The latter is a cumulative bias of the real measured values from the modelled cyclic line. These calculations made use of the BioDare2 package (https://biodare2.ed.ac.uk/) by setting the data input as "cubic dtr" and choosing the analysis method "mFourfit" (Zielinski et al., 2014).

3.3.3 Statistical analysis

We performed the comparison between mean values under the different environments, and for the different traits, using the JMP14 software (SAS Institute Inc., Cary, NC, 1989-2019). After verifying normal distribution of values, we used one-way ANOVA to examine significant differences between the Heat3.1$^{Hs/Hs}$; Heat3.1$^{Hv/Hv}$; Heat3.1$^{Hs/Hv}$ genotypes. For the circadian clock period, the delta between period of the same plants under high and optimal temperatures was compared between genotypes(Bdolach et al., 2019).

Since recombination rates among RNP-treated calli were not normally distributed, we compared these values between different treatments by using a non-parametric Kruskal-Wallis test (Ben-Israel et al., 2012).

3.2.4 Cas9/gRNA in vitro assays with cloned PCR products as template DNA

We prepared two plasmids, PL18.0015 and PL.0018, which carry the Hv and Hs parental sequences by amplifying the target region with specific primers PM18.0020 and PM18.0021 (Supplementary Table 1) and using DNA that we extracted from Barke and HEB-25-25.1, respectively. After Sanger verification of these two plasmids we used them to amplify 5' and 3' of the 178bp piece with the primers PM18.0002 and PM18.0024 (5' end), and PM18.0023 with PM18.0021 (3' end; Supplementary Table 1). We then tailored these 5' and 3' amplicons by a second PCR with PM18.0020 and PM18.0021 to generate 4 possible combination (2 parental and two recombinants; Supplementary Table 2). The amplicons were extracted from a gel with PureLink™ Quick Gel Extraction Kit (Thermo, cat. no. K210012) and cloned to pJET1.2/blunt with CloneJET PCR Cloning Kit (Thermo, cat. no. K1231). The plasmids were transformed to Stellar™ Competent Cells (Invitrogen, cat. no. 636766) using a standard heat shock protocol (30 min on ice, followed by 45 sec at 42°C, adding 250 µL LB and shaking at 37°C for an hour), followed by selection on LB agar plate with ampicillin. The Plates were incubated at 37°C overnight and colonies were verified by colony PCR with pJET1.2/blunt primers. Another verification was done by digesting plasmids with restriction enzymes XhoI & BsaI (NEB). Finally, the plasmids were sequenced, and the right order of the SNPs was confirmed.

The synthetic sgRNA composed of universal tracrRNA and unique crRNA, were ordered from Invitrogen (cat. no. A35512&A35507). The synthetic crRNA and tracrRNA was resuspended with 1x TE buffer to 100µM. We conducted the PCR

annealing of the crRNA and tracrRNA following the Invitrogen user guide protocol for a final crRNA:tracrRNA duplex concentration of 20 µM. We conducted *in vitro* assays following the BioLabs protocols. To obtain the best cleavage efficiency, the molar ratio of Cas9 sgRNA per target site was kept at 10:10:1 respectively. We included in a total volume of 24 µl reaction 3µl 10X Cas9 Nuclease Reaction Buffer, 3µl of 2000n (200nM final), 1µl of 6000nM *Streptococcus pyogenes* Cas9 Nuclease (New England Biolabs, cat no. M0386; 200nM final), and pre-incubate this for 10 min. at 25ºC. We then added 6 µl of plasmid (20nM final) , incubated the reaction for 15 min. more at 37°C, and stopped the digest by adding and mixing 1 ul of Proteinase K. The products and negative control (no sgRNA) were loaded on 1.5% agarose gel to test for linearization of plasmid.

3.2.5 RNP preparation and particle gold preparation

We adopted protocols from (Barcelo and Lazzeri, 1995) and (Sparks and Jones, 2009) for the delivery of RNP and plasmids into calli using gold particles. We weighed ten mg of BIO-RAD sub-micron gold particles (0.6µm) and added 1ml of 100% ethanol within a 1.5ml Eppendorf tube. We then sonicated sample for two min and pelleted particles by centrifugation at 13,000 rpm for 10 to 30 sec, and discarded supernatant. We repeated this ethanol twice more with vortexing and tap mixing instead of sonication.

We then added 1ml of sterile water to the tube and sonicated sample again for 2min and pulsed spinned in a microfuge for 3 sec, followed by supernatant discard. Finally, we resuspended gold particles with 500µl of sterile water, divided into 50µl aliquots and stored tubes at -20°C.

Assembly of the RNP was following Liang et al., 2018 . For ten reactions of RNP delivery we prepared a total mix of 100 µl including 4µl 10X Cas9 protein (20 µg), 20µl of sgRNA(20 µg) , 10 µg of 10X Cas9 reaction buffer, 66 µl RNase-free water and incubated this reaction at 25°C for 10 min. Prior to the use of golden particles we thawed the 50µl aliquots at room temperature and sonicated sample for 1-2 min. We then added this to the 100 ul RNP mix and gently yet thoroughly homogenized this by pipetting. We spread 15µl of the mixture onto the central region of each macro-carrier. The macro-carriers were air-dried on the benchtop at room temperature for 1h. The delivery of RNP into the calli cells that were positioned on

callus induction media followed the book “Transgenic Crops of the World: Essential Protocols - Google Books,” n.d.. However, we changed the loading volume for each shoot to 15-20µl vs. 5 µl in the original protocol and set the rapture disk to 1100 psi, and positioned the target cells (plate) 7cm below the stopping screen. After delivery of the RNP into calli we kept the plates for 48 hours in dark before harvesting for DNA extraction as a pool or individually.

3.2.6 Estimating recombination rate by Droplet Digital PCR (ddPCR)

The ddPCR reactions were conducted on the BioRAD QX200 Droplet Digital PCR System following manufacturer protocols (https://www.bio-rad.com/en-dz/product/qx200-droplet-digital-pcr-system?ID=MPOQQE4VY). Two primers and four distinct probes were designed; the first two probe are reporter fluorescent FAM and HEX attached to the first two SNPs of Heat3.1^{Hs} wild allele TTG: FAM attach to SNP1(**T**) and HEX to SNP2(**T**). In addition, two dark probes were also designed, to prevent attachment of FAM and HEX to SNP1 and SNP2 of Heat3.1^{Hv} cultivated allele **AC**A (Fig. 2). The Initial adjustments of the ddPCR reaction was making use of the four cloned plasmids that represent the four haplotypes (Supplementary Table 1). We made a serial dilution of the plasmid between 500 and 4 picogram/µl. We then mixed the plasmids to create three different assays: (1) **TT**G and **AC**A, (2) **TC**A and **AC**A, (3) **AT**G and **AC**A to generate the orange, blue and green clusters, respectively. It appeared that between 500-20 picogram/µl concentration, the plot result represented only a black cluster, considers all droplets as negatives. However, at 4 picograms, we could see the expected cluster distribution. Therefore, in the following ddPCR assays the plasmid diluted further to 12.5 femtogram/ul. Finally, we set channel one and two amplitude thresholds to 1051 and 2375, respectively, and used a concentration between 4 picograms/ul to 400 femtogram/ul since it was most suitable for plasmids detection with the expected distribution of the droplet clusters. For the ddPCR on the callus samples we measured DNA by nanodrop and used 10 ngr per reaction.

3.3 RESULTS

3.3.1 Design and in vitro examination of targeted PAM sites in Heat3.1 site

In this project we focused on two introgressions from the wild barley, each containing a QTL for different trait. These two QTL, namely *Hordeum spontaneum* Dry2.2 (HsDry2.2) and HsHeat3.1 reside on chromosomes 2 and 3 , respectively. The Heat3.1 served as the main locus to develop the toolkit to the design and execution of CAS9-mediated recombination. The parts describing the experiments done in the HsDry2.2 locus are summarized in section 3.2.5.

The main reason for selecting HsHeat3.1 is that in our field experiments we found the wild allele is causing pleiotropic effects, i.e. it accelerates the circadian clock output and also maintains more stable (significantly less reduction) grain yield under heat (Figure 1; Lazar et al. 2020).

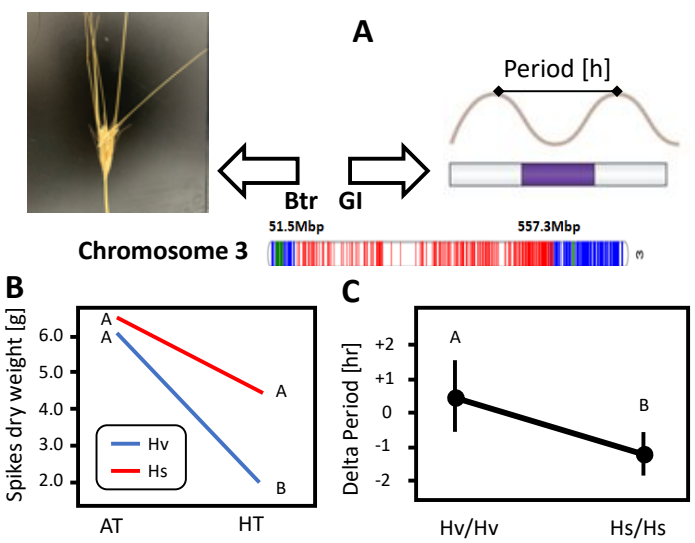


Figure 1. The genomic organization and phenotypic effects of the wild barley *Hordeum spontaneum* Heat3.1 locus. A) The wild introgression in line HEB-25-049, depicted in red, contains the Brittle rachis (Btr1 and Btr2) and GIGANTEA genes, which are involved in spike development and circadian clock rhythms, respectively. **B)** The *Heat3.1* locus is affecting the reproductive part under heat. Carriers of the wild (Hs) allele show less reduction of spike dry weight in transition from ambient to high temperature (AT to HT). **C)** Carriers of the wild allele show enhanced plasticity of the circadian clock rhythms under increased temperature. The delta in the clock period between high and optimal temperature is significant for carriers of the wild allele (Hs/Hs; mean acceleration of clock by 1.2 hr) as opposed to stable clock for those carrying the cultivated allele (Hv/Hv).

Following sequencing of an amplicon that identified 3 single-nucleotide polymorphic sites between the cultivated (*Hordeum vulgare* allele, hereafter Heat3.1^{Hv}) and wild (*H. spontaneum* allele; hereafter Heat3.1^{Hs}) barley genomes we designed probes and gRNA molecules. Figure 2 depicts the sequence organization including the SNP, the PAM sites and the primers in the relevant target site. This amplicon is in the vicinity (50K b upstream) to the barley ortholog of the GIGANTEA Arabidopsis gene (REF). We obtained the gRNA by assembling specific crRNA and a general tracrRNA following manufacturer protocols (Invitrogen, see Methods). The 18.0083 and 18.0086 synthetic gRNA molecules were assembled with CAS9 protein to RNP,

at the final molar ratio of 200nM(sgRNA):200nM(cas9):20nM(plasmid-template). We then incubated the RNP with the pJET plasmid, and both sgRNA molecules resulted in a specific digest and linearization of the plasmid (Fig. 1B).

Before continuing from these *in vitro* assays to *in vivo* application of these RNP to plant mitotic cells, we have engineered similar plasmids that contains the two parental alleles, i.e. Hv and Hs (Fig. 1B). Besides, we recombined these allelic fragments by “tailoring PCR” and generated eventually four pJET plasmids containing a piece of 178 bp harboring the four haplotypes based on 3 SNPs (Hv, Hs and two recombinant products) (Fig. 2B). These plasmids served as controls for measurements of recombination frequencies *in vivo*.

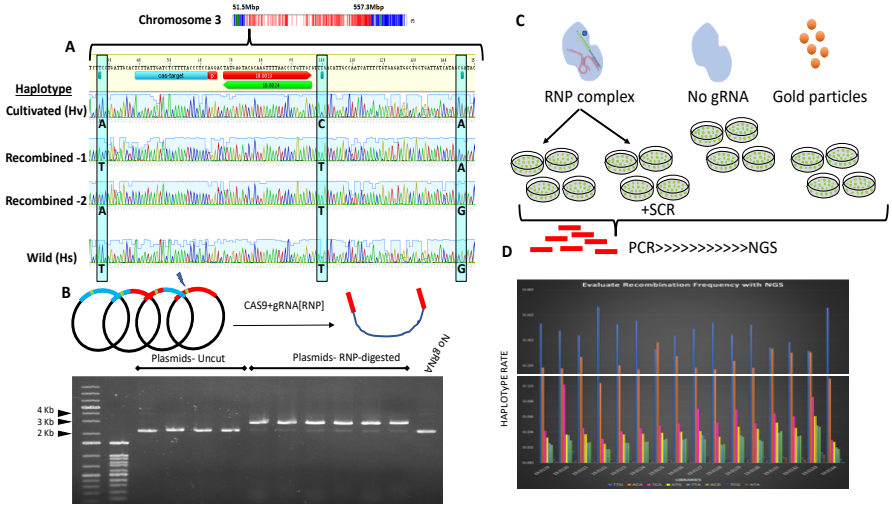


Figure 2. Haplotype organization, *in vitro* and *in vivo* assays for Cas9/sgRNA-mediated digestion and recombinogenic activity, respectively. A) The 178 bp including SNP 1 to 3 showing difference between Hs and Hv alleles. Above Sanger sequencing of the cloned amplicons indicated the Protospacer adjacent motif (*PAM*) in red and upstream sequence used as cRNA with tracrRNA to build the sgRNA. Downstream to these (green and red arrows) are the forward and reverse primers used to tailor the recombined alleles. **B)** The 178 bp were amplified and cloned into pJET plasmids and tested in vitro for specific digest. **C)** Calli of mixed homozygous and heterozygous calli were treated with RNP and controls, followed by PCR amplification of the 178 bp and **D)** Next generation sequencing analysis to determine the rate of the different haplotypes. Color coding for the parental (ACA and TTG) and other possible recombined haplotypes indicated below bar plot.

The RNP complex including the 18.0083 sgRNA located between the first and second SNPs**,** which we validated in the *in vitro* assay, delivered by gold particle bombardment into calli cells (Fig. 2C) . These calli were originated from mature embryos segregating for the *Heat3.1* locus (self of heterozygous plant in the BC1S5 generation (Maurer et al., 2015)). For the most parts, biolistic delivery followed Liang et al., 2018 with few modifications. We divided the first experiment into six treatments (**I-VI**) that included three replicates of ten calli in each plate. All samples contained the two components of RNP complex Cas9 protein with sgRNA, except for

the negative control (**VI**) which contained only the Cas9 protein. Besides, to the RNP complex groups **I** and **II** we performed co-bombardment of a plasmid containing 'Helper morphogenic genes' BABYBOOM WUSCHEL in the thought to achieve accelerated cell divisions and theoretically increased recombination rates(Svitashev et al., 2016) (Lowe et al., 2016). We added to the media of groups **II** and **IV** the SCR7 substance. The SCR7 is an inhibitor for the non-homologous end joining (NHEJ) process (Ma et al., 2016)(Chu et al., 2015)(Maruyama et al., 2015). Group **V** incubated with high osmotic media one day before the bombardment (Supplementary Fig. 1A). The bombardment followed by PCR-based library preparation for next-generation sequencing to estimate recombination rates. This experiment included eighteen libraries (six treatments X 3 replicates). DNA extraction and PCR amplification with a barcode-linker were conducted for each library to allow multiplexing. The amplicon libraries analyzed by MiSEQ sequencing using 150bp paired-end (PE150) (Supplementary Table 1). Besides, we included the four cloned plasmids with parental and recombined alleles (Fig. 1b) as a control template for the PCR reactions and the NGS.

We analyzed NGS results for both HDR and NHEJ. As expected, the cloned plasmids showed only one dominant allele, with a low and neglected rate of the other alleles. We identified the HDR products by scoring the different alleles and their frequency; TTG and ACA are the parental Heat3.1^{Hs} and Heat3.1^{Hv} alleles, respectively. The four main recombinant products are TCA, ATG, TTA, and ACG. TCA and ATG generated from DSB between SNP1 and SNP2 and TTA and ACG from DSB between SNP2 and SNP3. Besides, TCG, ATA correspond to double recombination, i.e. one between SNP1 and SNP2 and second between SNP2 and SNP3. We observed recombination in all the different treatments with a higher frequency of TCA and ATG recombinants. However, we could not identify a significant change between the treatments with regard to the proportion of the recombinant compared to the parental alleles. Since the negative controls showed similar recombination rates as the other treatment, the bombardment repeated with more negative controls replicates, to refute, for example, the possibility that the negative control also contained the sgRNA as the examined samples.

In the second bombardment, we mainly wished to understand why we found recombination frequency also in the negative controls. This experiment included four

treatments, and each treatment had four replicates, together we had sixteen plates with ten calli in each one. The RNP group divided into two, four plates with SCR7 and four plates without SCR7. Two negative control treatments included Cas9 protein without sgRNA or only gold particles (Fig. 2C). Again, analysis of the NGS results indicated that under this experimental set-up, where each sample composed of segregating calli (homozygous and heterozygous) recombination rate is on average 4.8%, yet not significantly different between RNP and negative controls (Fig 2D).

3.3.2 *mitotic recombination mediated by RNP and analysis by digital droplet PCR (ddPCR)*

Realizing this chimeric issue resulting from PCR and its possible causes, we switched to digital droplet PCR (ddPCR) as the detection method. One underlying assumption of dPCR is that it includes random distribution of DNA molecules into the partitions and amplification of a single target in a partition (Whale et al., 2013). In our study, this would be either the parental or the recombinant alleles in single droplets. In these ddPCR assays, two probes are the reporter fluorescent FAM and HEX attached to the first two SNPs of Heat3.1^{Hs} *H. spontaneum* (Hs) allele TTG: the FAM attach to SNP1(**T**) and HEX to SNP2(**T**). Besides, we designed two dark probes to prevent attachment of FAM and HEX to SNP1 and SNP2 of Heat3.1^{Hv} cultivated allele **AC**A (Fig. 3a). Once recombined, the two probes are changing from CIS to TRANS configuration (Fig. 3b). Consequently, FAM and dark probe attach to **TC**A recombinant and HEX and dark probe attach to **AT**G recombinant (Fig. 3b).

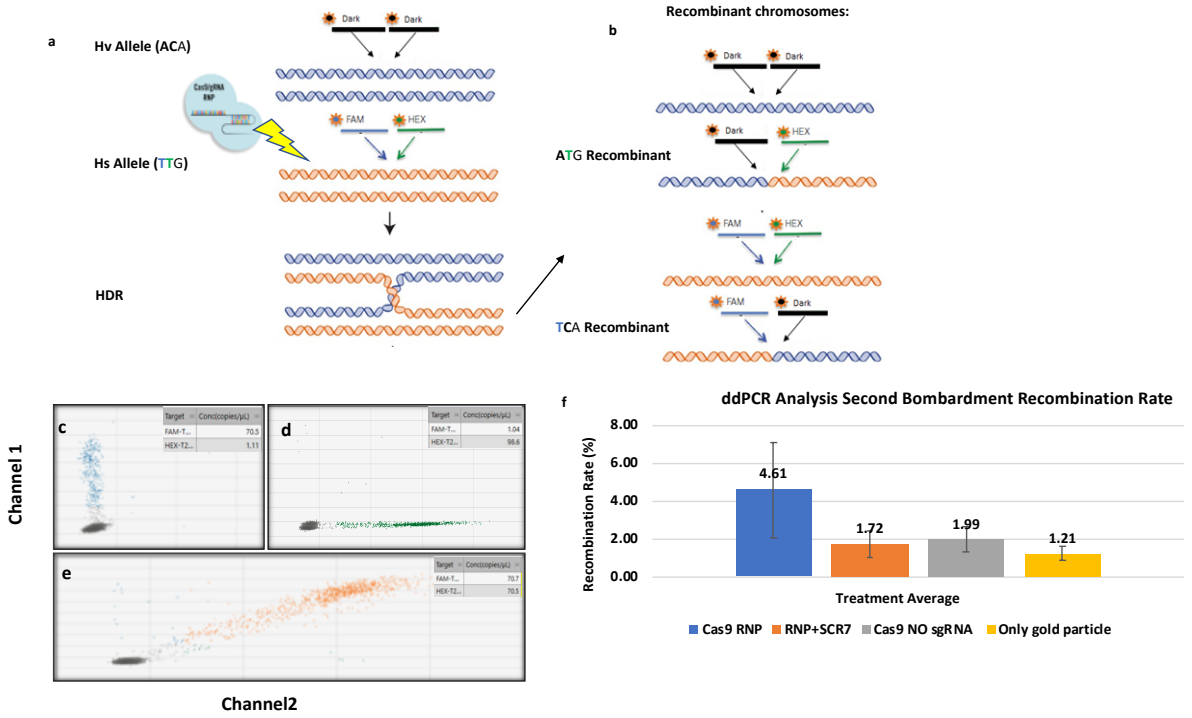


Fig. 3 Droplet digital PCR analysis assay for wild type and recombinant alleles. **a)** The FAM and HEX probe attach to the wild (Hs) SNPs, while two dark probes attach to the complementary cultivated (Hv) SNP **b)** DSB generated by RNP followed by HDR will generate two recombinant products. **c)** With no recombination, FAM and HEX probes attach to Hs allele and visualized only as orange cluster, while the dark probes attach to Hv allele and create black cluster. When some of the droplets includes recombinant template for the ddPCR, this FAM and dark probe, which attach to **T**CA recombinant, will generate blue cluster. Similarly, HEX and dark attach to **A**TG will generate green cluster. ddPCR using plasmids **c)** **T**CA& **AC**A templates **d)** **A**TG& **AC**A templates and **e)** **TT**G& **AC**A templates. Copies of target per microliter (copies/ul) are represent in the upper right insets. **f)** Mean percentage of recombination rates at the second bombardment. Y-axis is [(FAM-HEX)/HEX]*100 absulte value, indicating the recombination events with eror bars. Each treatmnet include four replicate with ten calli each.

Initially, in these ddPCR experiments for the in vivo assays we estimated the recombination events by subtracting concentration of the FAM probe (copies/µL) from the HEX probe, and divided this delta by the smaller number. Based on these calculations the calli that undergone Cas9 RNP treatment had higher recombination frequency than the other treatments (Fig. 3f). The negative results and particularly Cas9 without sgRNA treatment showed less than half of recombination frequency in the RNP treated calli (Fig. 3f). However, variation within treatments was relatively high, and differences between means were not significant according to parametric or non-parametric tests. We figured out that these high values of variation originated from the heterogeneity of the calli samples (homozygous and heterozygous DNA extracted in bulk). Therefore, in the third bombardment, the experiment divided to four treatments, with each represented by six replicates (plates, with 20 calli in each): RNP complex,

Cas9 expression cassette, Cas9 protein with no sgRNA, and only gold particle (Fig. 4a). To avoid heterogeneity (mix of heterozygous and homozygous samples) and derived noise in ddPCR we scaled down DNA extraction methods. We used the TaqMan®SNP assay to distinguish between individual homozygote and heterozygous calli (Fig. 4B). We analyzed only heterozygote calli in the ddPCR for recombination events.

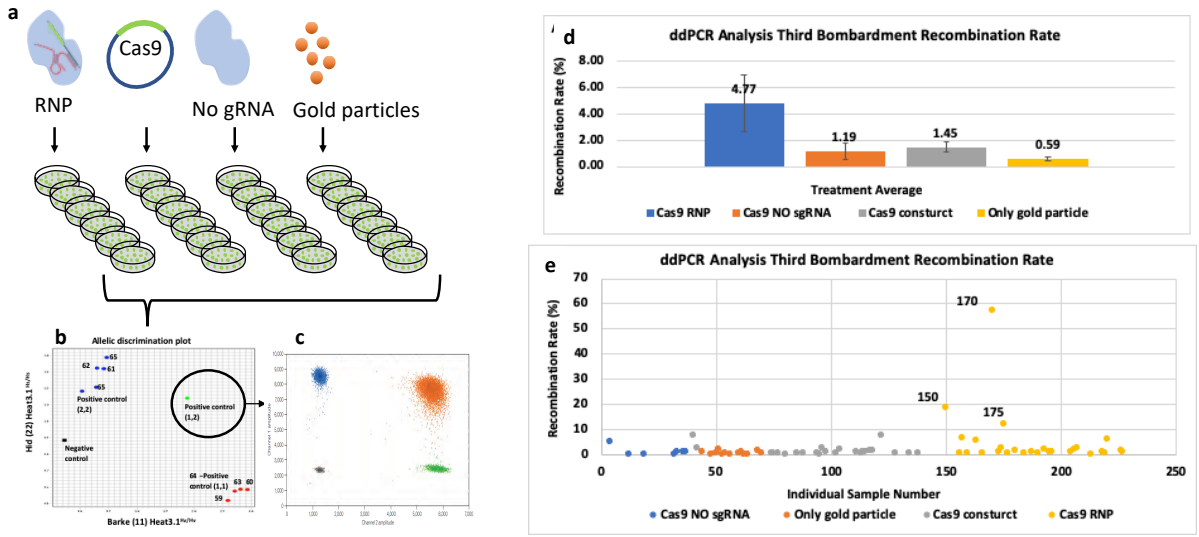


Figure 4. RNP delivered into calli heterozygous for the *Heat3.1* locus increases recombination rate. Calli originated from mature seeds segregating for the *Heat3.1* locus were **a)** treated with RNP or CAS/sgRNA plasmid contain the sgRNA between SNP1 and SNP2, followed by **b)** TaqMan®SNP assay to distinguish between the homozygotes to the heterozygotes, of which **c)** the later analyzed by ddPCR for recombination rates. **d)** A significant higher recombination rate in the Cas9 RNP (4.77%) treatment Y-axis is [FAM-HEX/HEX]*100 absulte value, indicating the recombination events with eror bars. Each treatmnet include four replicates with ten calli in each. **e)** Distribution of the recombination rate among individual calli samples as measured by ddPCR. Note the extreme high recombination rate found in several of the RNP-treated calli albeit the non-uniform penetrance of the treatment.

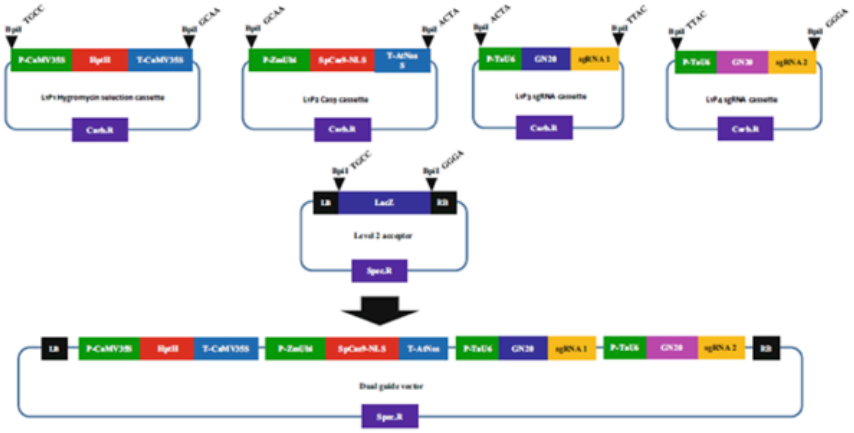
Similar to the second bombardment (Fig. 3D), in this third bombardment with ddPCR analysis we identified higher recombination rates in the RNP treatment (4.77%) compared to the other treatment (Fig. 4d). Indeed, averaging of the different samples in each treatment showed a decreasing of the noise in all samples including negative controls (Cas9 without sgRNA and gold particle only) as reflected in the error bars (Fig 4c). Moreover, on average, there was a significant change in the average and distribution of recombination rate between RNP-treated to other treatments (Kruskal-Wallis test; $P<0.0081$), indicating that RECAS9 worked.

Profoundly, at this point, there seem to be differential penetrance of the RECAS9 to the different samples, with some samples showing no recombination, while others are

reaching 57% (sample 170; Fig. 4e). To examine if this is originated from lack of penetrance of the RNP into cells or lack of efficiency in CAS9-gRNA that we selected we also performed RECAS9 in heterozygous calli using agrobacterium mediated genome editing (see 3.2.3).

3.2.3 *mitotic recombination mediated by Agrobacterium and analysis by digital droplet PCR (ddPCR)*

We have used the same gRNA sequence to build a binary vector that includes CAS9 and the gRNA driven by QBIQUITIN and U6 promoter from wheat (REF). The design and build of the binary construct was following protocols described in Bartlet et al. (2018). This binary vector (Fig. 5) was transferred to Agrobacterium strain AGL1 and delivered into 4-5 days old calli by incubation. Next, DNA was extracted from these calli cells, followed by genotyping to identify heterozygous Heat3.1$^{Hs/Hv}$ individuals. These were analyzed by ddPCR and compared to control heterozygous incubated with AGL1 with no vector.



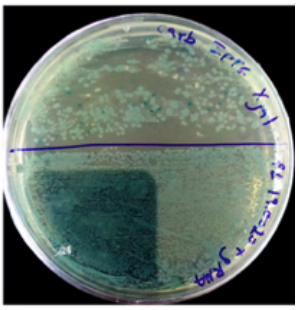
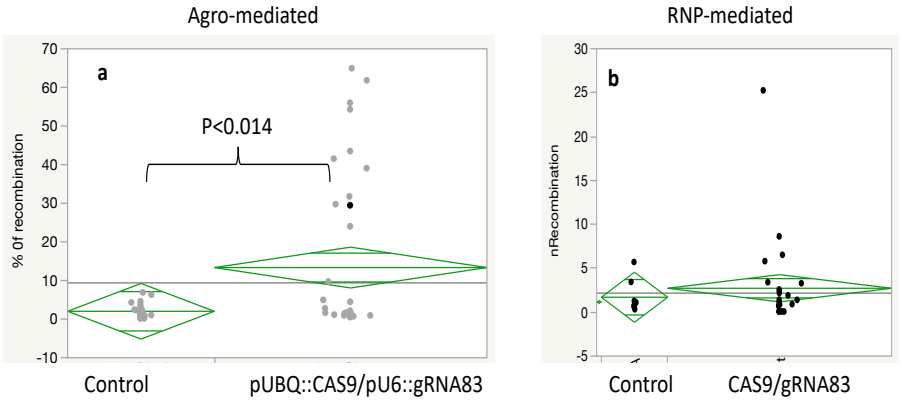


Figure 5 Agrobacterium mediated RECAS9 in Heat3.1 locus.

At this point we changed the way we calculate recombination. We made this change following the advice of Eddy vonColenburg (BioRAD specialist for ddPCR). In this analysis the recombination is calculated only for samples that obtained at least 100 positive droplets, and it based on the complementary to the linkage values given by the Quantasoft (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/QuantaSoft-Analysis-Pro-v1.0-Manual.pdf). Figure 6 depicts the comparisons between RNP- and Agrobacterium-based RECAS9. Notably, recombination rate in the Agro treated heterozygous calli was significantly higher than control (13.34 vs 2.2%; P<0.014; Fig 6a). Moreover, with the new calculation the penetration, which is defined as the

percentage of calli with recombination level above 5% is doubled for the Agrobacterium treatment (30.7%) compared to the RNP experiments (14.8%; Fig. 6b) Therefore, the new mean value of recombination among RNP-treated calli (2.72%) is significantly lower than Agro treated calli.

Figure 6. Agro-mediated REACS9 shows significant effect on recombination rate with higher penetrance compared to RNP. Calli originated from mature seeds segregating for the *Heat3.1* locus were treated with **a)** CAS/sgRNA plasmid contain the sgRNA between SNP1 and SNP2 or **b)** by RNP using same gRNA in a synthetic form. Following TaqMan®SNP assay only single heterozygous calli DNA (Fig. 4b) was later analyzed by ddPCR for recombination rates.



3.2.4 Designing and testing alternative CAS9 proteins for targeting mitotic recombination

One attempt we have been doing to increase recombination is by fusion of Cas9 RNP complex with SYN4 protein. SYN4 (a-kleisins) is one of the core component of mitotic Cohesin complexes in Arabidopsis (Jiang et al., 2007). Example of similar work was done by Sarno et al., 2017 which fused SPO11 protein (belonging to the meiotic complex) to different DNA binding module for programming meiotic crossovers.

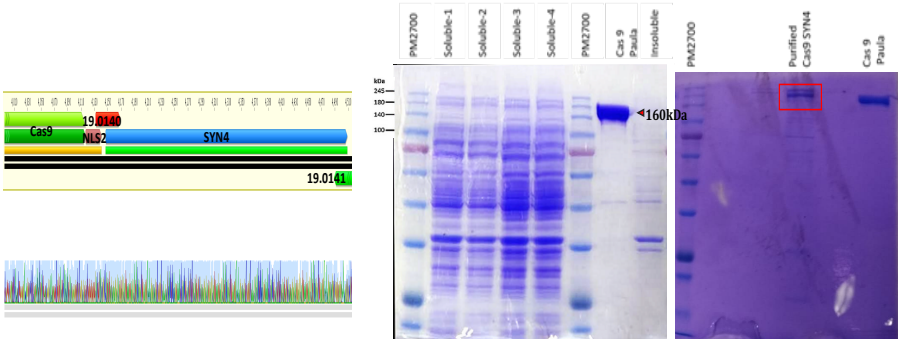


Figure 7. Design and heterologous expression of CAS9-SYN4 enzyme. a) Sanger sequencing validation of the fused SYN4 Cas9. The design is shown for the fusion point on the upper level. Cas9 in green, NLS2 in red, 19.0140FWD primer red and 19.0141 REV green, SYN4 gene blue. The sequencing chromatograms are shown on the bottom. **b**) expression and purification of Cas9 SYN4 by Ni-*NTA*. The purified products were analyzed by 20% SDS-PAGE gel. Control was purified CAS9 from Weizmann Institute (lane7) and the remaining lanes depict different stages of purified Cas9 SYN4 protein. **c)** Final stage of the purified Cas9 SYN4, two potential bands are visualized (marked with red square, lane 4), and these were sent for protein sequencing for validation 3-color protein marker PM2700 (cat-PM2700) used as a protein ladder with molecular weights ranging between 5 to 245kDa (245, 180, 140, 100, 75, 60, 45, 35, 25, 20 kDa) (lane 1, 6).

Following the purification and validation of the purified CAS9-SYN4 protein by LC-MS we have scaled up the manufacturing of the enzyme with the protein expression unit in Weizmann Institute. Testing this enzyme in vitro with plasmids comtaining the target PAM showed similar digest activity as we observed for commercial CAS9 (Fig. 2b). This enzyme is currently used in RNP experiment to compare efficiency of editing and recombination compared to the regular CAS9.

3.3.5 RECAS9 in Dry2.2- design, experiments and complications

In a similar manner to that we conducted for the HsHeat3.1 we identified two near-by SNP within the Dry2.2 QTL for drought resilience (Merchuk et al. 2018). Figure 8 depicts the organization of the locus and the relevant primers and PAM sites that we targeted for DSB and recombination using two gRNA. Also in this locus we performed Agro- and RNP-based experiments. The gRNA proved to be effective in targeting DSB in-vitro with plasmids cloned with the pAM sites (data not shown).

Unlike the experiments with Heat3.1 neither the agro-mediated nor the RNP experiments achieved a significant recombination rate for the heterozygous calli. A recent paper that came out could explain this lack of recombination. In the Pan genome analysis of barley it was discovered that the HvCEN that resides within the HsDry2.2 (Merchuk et al. 2018) is part of a chromosomal inversion that occurred during evolution (Fig. 9a). Inversion are known to prevent recombination between sister chromosmes in meiosis. The wild allele for the HsDry2.2 is originated from HEB-04-096 and is oart of the HEB multiparent population (Maurer et al. 2015). To examine this line we used the DNA of the segregating calli with the TaqMan probe first, and then with the primers that detect the inversion (Jayakodi et al., 2020). Indeed, we found a full co-segregation of the inversion with the Hs allele (Fig. 8).

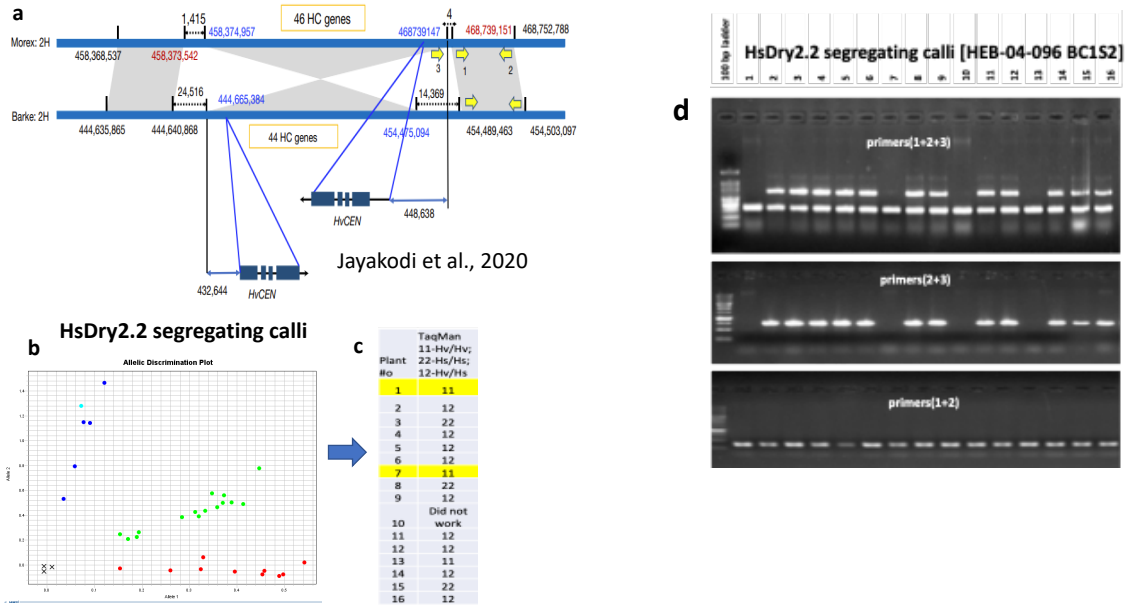


Figure 8. Chromosomal inversion in the HsDry2.2/HvCEN region prevents recombination. **a**) Genomic organization and inversion between the Morex and Barke lines

(the Barke is the Hv background for HsDry2.2). **b)** TaqMan®SNP assay to distinguish between the homozygotes to the heterozygotes, as summarized in **c)** genotyping of 16 segregating calli (11, homozygous for Barke allele; 12, heterozygous for Barke and wild alleles; 22, homozygous for wild allele). **d)** testing segregating calli shows complete cosegregation between Barke allele and inversion suggesting this prevents recombination with allele of wild barley originated from HEB-04-096.

These recent findings prompt us to follow a different trajectory in using RECAS9 to fine-map the causal diversity in HsDry2.2 (see discussion 3.3.3).

3.4 DISCUSSION

3.4.1 The relevance of RECAS9 for functional analysis

The most common way to test function of a gene is by generating mutations or knockouts (KO) in the gene and testing consequences on the phenotype of interest. In that regard, in a very short time since its first use in plants (REF) CRISPR has become the method of choice to mutate candidate genes to test their function. However, more recent and sophisticated use of CRISPR in crop plants show that perhaps the more relevant variation to be manipulated does not lie in the gene body itself. (Rodríguez-Leal et al., 2017) demonstrated that CRISPR/Cas9 genome editing of promoters generates diverse cis-regulatory alleles that provide beneficial quantitative variation for breeding. They devised a simple genetic scheme, which exploits trans-generational heritability of Cas9 activity in heterozygous loss-of-function mutant backgrounds, to rapidly evaluate the phenotypic impact of numerous promoter variants for genes regulating three major productivity traits in tomato: fruit size, inflorescence branching, and plant architecture. Similarly, Liu et al. (2021) have recently engineered quantitative variation for yield-related traits in maize by making weak promoter alleles of CLE genes, and a null allele of a newly identified partially redundant compensating CLE gene, using CRISPR–Cas9 genome editing. These studies implicate that KO are not necessarily the best choice, or at least not the only one, to link between cause and effect when it comes to quantitative phenotypes. In that regard, having the ability to pinpoint recombination of wild alleles becomes crucial to avoid just “looking under the streetlight”, i.e. thinking that the gene itself is the one making the difference. This was also true in the case of the first QTL cloned in plants, in which two invertase (LIN5 and LIN7) could be the gene underlying sugar content yield in tomato, and recombinants in that region could resolved this to LIN5 only.

3.4.2 The efficiency of RECAS9 for generating transgene-free recombinant plants

The results of this project indicate that CRISPR-Cas9 could direct recombination and allow identification of causal variation regardless of genes nearby. The question is though how many events will be required to achieve such fine mapping? As with classic map-based cloning this is off course depending on resolution of the primary genome scan, the effect of the causal variation and its complexity (e.g., is it dependent on epistatic interactions), among other considerations. The fact that we are able to achieve directed recombination is only the first step towards RECAS9-based trait mapping. Another question is the efficiency to deliver the recombination events in mitosis to the next generation of recombinant genotypes. One major bottleneck downstream this is the ability to regenerate recombinant plants in an efficient manner and this relies on efficient cell culture pipelines. In barley, we currently manage to achieve 10-20% regeneration from mature Golden promise embryos and approximately half of that for other genetic backgrounds such as Barke and Noga (data not shown). However, we still do not know how many of those embryos that acquire the RNP and get edited will inherit this to a stable recombinant plant. Our results support previous observations that show feasibility of RNP to achieve edited plants, if we follow a penetration to calli of app. 15 then app. 15 recombinant plants out of 1,000 could be expected with RNP. With agrobacterium-based RECAS9 these values would increase dramatically owing to selection in the process and better penetration as we observe in our experiments (Fig. 6).

In previous studies that used RNP to generate mutations in wheat (they observed the mutation frequencies of the three homoeologs that were targeted with same gRNA (*TaGW2-B1*, *-D1* and *-A1*) were 2.2% (14 plants out of 640 treated immature embryos), 4.4% (28/640) and 0% (0/640), respectively. So, for KO mutations the RNP is feasible and reasonable and has the advantage of producing fewer off targets probably due to the transient nature of the complex as compared to stable transformation. However, in RECAS9 the events are not mutation but rather derived recombination event and in mitotic cells, which without a selective agent as GFP in yeast (Sadhu et al. 2016) are expected in lower frequencies. Analysis of T0 plants that we currently grow by NGS should tell us if these calculations are valid, or that reduction is expected due to chimeric nature of plants.

How could we then improve RNP based RECAS9? In previous studies There are at least two directions that we currently follow to increase the efficiency: 1) by automating the embryo extraction and therefore increase number of treated calli (in collaboration with Ron Bernstein, ARO, and funded by Chief Scientist Office at Ministry of Agriculture) , and 2) by developing more efficient delivery nano-vehicles for RNP (in collaboration with Dr. Markita Landry, funded by BSF-NSF).

3.4.3 Macro and micro RECAS9 for dissecting HsDry2.2, a rare wild QTL with proven yet unknown causal effects on yield stability under stress

In the beginning of this project HsDry2.2 seemed as a target QTL that represents low recombingenic region owing to its percentric position. However, the fact that the two alleles of Barke and HEB-04 are differentiated by historic inversion under domestication (Jayakodi et al., 2020) strongly suggest that this is the cause for reduced recombination as we observe in our experiments. Therefore, we already began to explore the use of additional HEB lines without inversion and no significant phenotypic effect to allow micro-RECAS9, i.e. inducing recombination in heterozygous plants. At the same time, the current large amount of heterozygous seeds that were generated between Barke and HEB-04-096 will be used for macro-RECAS9, or for reversing the inversion as was recently shown for Arabidopsis knob gene (Schmidt et al., 2020). Achieving such inversion would further allow us to realize if variation within or outside HvCEN underlie its unique contribution to resilience under drought (Merchuk et al., 2018).

CONCLUSION

This project supports the utility of RECAS9 for directing recombination in a relevant crop plant. The recombination values obtained at and the methodologies used (e.g. single callus ddPCR and RNP delivery) should guide us in the continuation of this project. It will also allow a rationalized design for integrating engineering and nanotechnology solutions to improve the efficiency and utilization of RECAS9. At this point it appears that the use of Agrobacterium-based delivery for the sgRNA and the CAS9 is more efficient than using RNP and additional efforts should be made to deliver RNP in an efficient manner.

3.5 APPENDIX

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3.5 DISSEMENATION

Lazar S, Bishara K, Bdolach E , Merchuk-Ovnat L, Shental N, Sherman A, Fridman E (2018) RECAS9: Recombining and Fine-Mapping QTL Alleles by Genome Editing. In: International Plant Molecular Biology , Montpellier, France. (Poster)

Shelly Lazar, Manas Ranjan Prusty, Khaled Bishara, Amir Sherman, Eyal Fridman (2020) RECAS9: Recombining wild species introgression via mitotic gene editing in barley bioRxiv 2020.01.07.897280; doi: https://doi.org/10.1101/2020.01.07.897280

Manas Ranjan Prusty, Shelly Lazar, Khaled Bishara, Amir Sherman, Eyal Fridman (2021) RECAS9-Mediated QTL mapping by Mitotic Genome Editing in Cereals. In: Plant Genome Engineering: From lab to field, Denver, USA (Keystone eSymposia). (Invited short talk)

Manas Ranjan Prusty, Shelly Lazar, Orit Amir-Segev, Amir Sherman, Eyal Fridman (2021) RECAS9: Recombining and Fine-Mapping QTL Alleles by Genome Editing. In: Plant Genome Engineering: From lab to field, Denver, USA (Keystone eSymposia). (Poster)