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Brief Communication

In vivo maternal haploid induction in tomato

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Tomato (*Solanum lycopersicon*) is the second largest vegetable crop and the largest fruit crop (Costa and Heuvelink, 2018). Progress in tomato breeding has been achieved by classical breeding, introgression of traits found in related wild *Solanum* species and exploiting heterosis in F_1 hybrid crosses (Lin *et al*., 2014). These approaches require the development of inbred lines to reduce or largely eliminate heterozygosity. Classically, multiple rounds of selfing or backcrossing are used to generate inbred lines (Gale, 1980), but homozygous lines can also be obtained in a single generation using doubled haploid (DH) technology (Jacquier *et al*., 2020). However, tomato is highly recalcitrant for haploid induction (HI) (Seguí-Simarro, 2010).

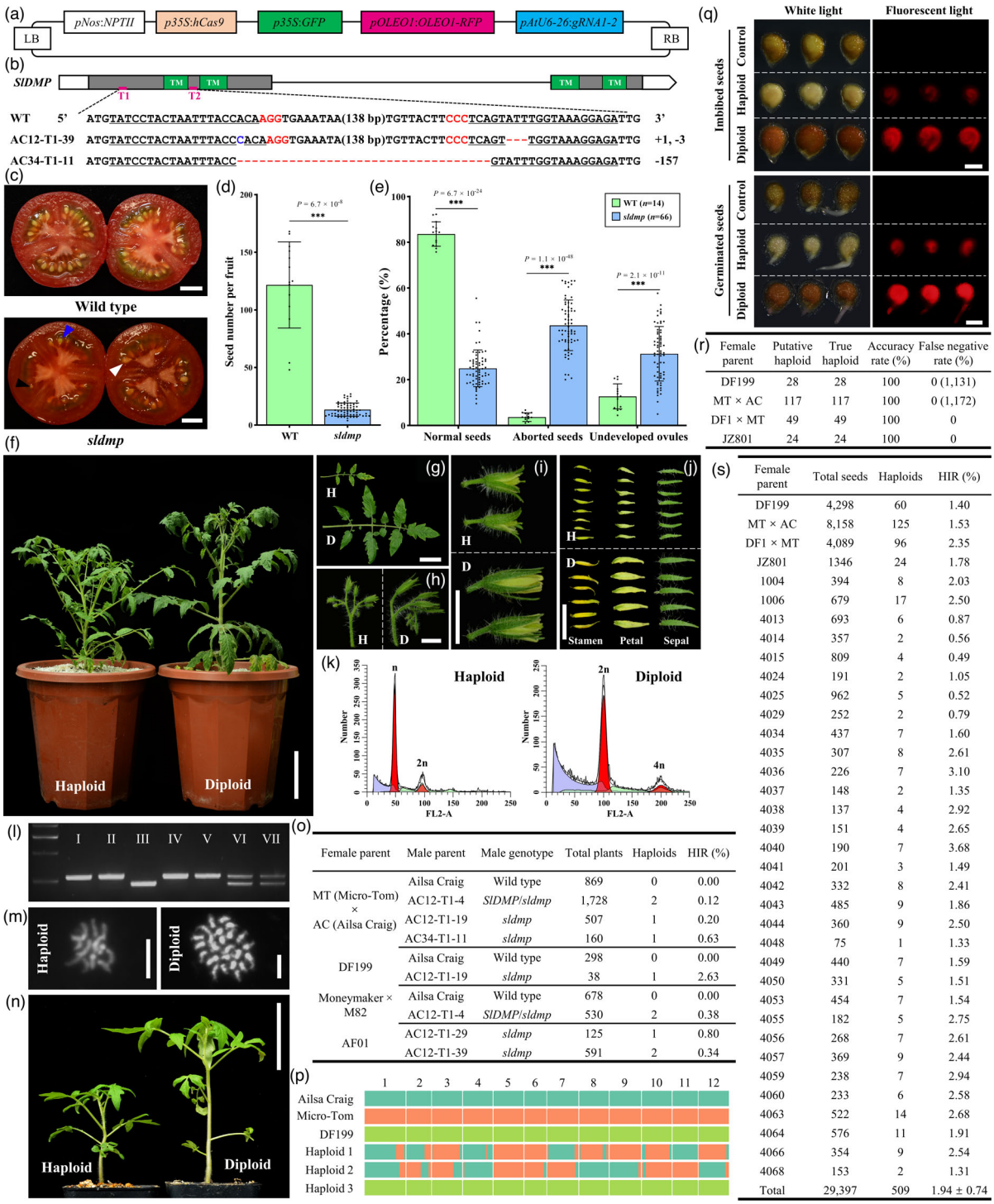
Significant breakthroughs in DH production were made after identification of the *zmpla1/mtl/nld* and *zmdmp* mutant genes that induce *in vivo* maternal haploid embryos in maize and extension of this technology to other monocot crops (Jacquier *et al*., 2020 and references therein). The utility of *dmp* mutants for HI in dicots was demonstrated in *Arabidopsis* (Zhong *et al*., 2020), but it is not known whether this approach can be applied in dicot crops. Here we show that *dmp* mutants can also be used for efficient and genotype-independent maternal HI in tomato.

The tomato genome contains a single *DMP* gene (*SlDMP*; Solyc05g007920) that is highly expressed in pollen and flower buds (Zhong *et al*., 2020). We generated *sldmp* mutants in cv. Ailsa Craig using a CRISPR-Cas9 mutagenesis construct that includes the FAST-Red marker for haploid identification (Zhong *et al*., 2020, Figure 1a). Homozygous or biallelic *sldmp* mutants with insertions and/or deletions that resulted in translational frame shifts and premature stop codons were generated (Figure 1b). Compared with wild type, *sldmp* mutants significantly reduced the number of filled seeds (Figure 1c,d) and increased the percentage of both aborted seeds and undeveloped ovules (Figure 1e), as previously reported (Zhong *et al*., 2020).

We determined whether *sldmp* mutants can induce maternal haploids after selfing. In the absence of segregating molecular markers, we first identified putative haploid plants based on their phenotype, that is, smaller organs and sterility (Zhong *et al*., 2019, 2020). Among 55 T_1 seedlings, one plant (Figure 1f) showed the typical haploid phenotype (Figure 1g–j). This plant was confirmed to be a true haploid by flow cytometry (Figure 1k). Our data suggest that *sldmp* mutation facilitates *in vivo* haploid embryo development in tomato.

Next, we crossed four wild-type F_1 female plants (listed in Figure 1o) from different genetic backgrounds with *sldmp* mutants to determine whether *sldmp* pollen can also induce maternal haploids upon outcrossing. Ten haploids derived from these crosses were first screened by molecular markers and confirmed by chromosome counting, flow cytometry and plant phenotype (Figure 1l–o). To confirm their maternal origin, three of these haploid seedlings were used for whole-genome resequencing. Single-nucleotide polymorphism (SNP) analysis showed that none of the seedlings carried paternally derived SNPs, suggesting that *sldmp* induces 'clean' maternal haploids (Figure 1p).

The *Arabidopsis* FAST-Red marker is expressed in the embryo and endosperm and can be used in crosses to distinguish between diploid seeds derived from double fertilization (marker expression in the embryo and the endosperm) and maternal haploid seeds (marker expression only in the endosperm) (Zhong *et al*., 2020). To determine whether FAST-Red can be used to identify tomato haploids, we analysed FAST-Red expression in seeds from a DF199 × *dmp* cross. Imbibed seeds were first classified into red and white seed groups based on their colour under white light. Under fluorescent light, the red seeds showed weak RFP expression in the endosperm and strong RFP expression in the embryo, while 70% of white seeds showed weak RFP expression in the endosperm and no RFP expression in the embryo (Figure 1q). Some white seeds showed RFP expression in the embryo and endosperm under florescent light and were recategorized as red/RFP-expressing seeds. These two groups were confirmed by checking root tip RFP expression (Figure 1q). The embryos in red seeds were scored as putative diploids, while the white seeds with weak RFP expression in the endosperm and no RFP expression in the embryo were scored as putative maternal haploids. Ploidy analysis of 218 putative haploid and 2303 putative diploid seedlings showed that FAST-Red can be used with 100% accuracy for selection of maternal haploids in tomato (Figure 1r).



(r)

Female parent	Putative haploid	True haploid	Accuracy rate (%)	False negative rate (%)
DF199	28	28	100	0 (1,131)
MT × AC	117	117	100	0 (1,172)
DF1 × MT	49	49	100	0
JZ801	24	24	100	0

(o)

Female parent	Male parent	Male genotype	Total plants	Haploids	HIR (%)
MT (Micro-Tom) × AC (Ailsa Craig)	Ailsa Craig	Wild type	869	0	0.00
	AC12-T1-4	*SlDMP/sldmp*	1,728	2	0.12
	AC12-T1-19	*sldmp*	507	1	0.20
	AC34-T1-11	*sldmp*	160	1	0.63
DF199	Ailsa Craig	Wild type	298	0	0.00
	AC12-T1-19	*sldmp*	38	1	2.63
Moneymaker × M82	Ailsa Craig	Wild type	678	0	0.00
	AC12-T1-4	*SlDMP/sldmp*	530	2	0.38
AF01	AC12-T1-29	*sldmp*	125	1	0.80
	AC12-T1-39	*sldmp*	591	2	0.34

(s)

Female parent	Total seeds	Haploids	HIR (%)
DF199	4,298	60	1.40
MT × AC	8,158	125	1.53
DF1 × MT	4,089	96	2.35
JZ801	1346	24	1.78
1004	394	8	2.03
1006	679	17	2.50
4013	693	6	0.87
4014	357	2	0.56
4015	809	4	0.49
4024	191	2	1.05
4025	962	5	0.52
4029	252	2	0.79
4034	437	7	1.60
4035	307	8	2.61
4036	226	7	3.10
4037	148	2	1.35
4038	137	4	2.92
4039	151	4	2.65
4040	190	7	3.68
4041	201	3	1.49
4042	332	8	2.41
4043	485	9	1.86
4044	360	9	2.50
4048	75	1	1.33
4049	440	7	1.59
4050	331	5	1.51
4053	454	7	1.54
4055	182	5	2.75
4056	268	7	2.61
4057	369	9	2.44
4059	238	7	2.94
4060	233	6	2.58
4063	522	14	2.68
4064	576	11	1.91
4066	354	9	2.54
4068	153	2	1.31
Total	29,397	509	1.94 ± 0.74

Figure 1 *sldmp* mutants induce maternal haploid seeds. (a) The CRISPR/Cas9 mutagenesis vector targeting *SlDMP*. (b) Schematic representation of *SlDMP*. Green blocks, transmembrane domains (TM); pink lines, the targeted regions (T1, T2). (c) Fruits from selfed plants. White, black and blue arrowheads, indicate normal seeds, aborted seeds and undeveloped ovules respectively. (d,e) Quantification of seed number (d) and seed phenotypes (e) in fruits shown in (c). Data represent the mean ± SD; ***$P < 0.001$ (two-tailed Student's *t*-test); *n*, number of fruits. (f–j) Haploid (H) and diploid (D) phenotypes. (k) Flow cytometry-based ploidy verification. (l) Seedlings from putative haploids were genotyped with molecular markers. Left lane, DNA size marker; I–VII, PCR bands of the DF199 hybrid, AF01, a *sldmp* mutant, a haploid from DF199, a haploid from AF01, DF199 × *sldmp* and AF01 × *sldmp* respectively. (m,n) Images of haploid and diploid chromosome sets (m) and seedlings (n). (o) Outcrossing HIR of *sldmp* mutants from the T_1 generation. (p) Recombination maps constructed via a sliding window approach across all 12 tomato chromosomes for 3 haploids and their corresponding parents. Haploids 1–3 were derived from maternal parents MT (Micro-Tom) × AC (Ailsa Craig), MT × AC and DF199, respectively. (q) FAST-Red-based haploid seed identification. (r) Accuracy of FAST-Red-based haploid identification. (s) Outcrossing HIR of *sldmp* mutant from the T_2 generation. Female lines indicated by numbers are breeding lines developed by Wencai Yang and Huolin Shen (China Agricultural University). Scale bars: 5 μm (m), 2 mm (p), 1 cm (c, h, i and j), 5 cm (g and n) and 10 cm (f).

Next, we used FAST-Red marker for haploid seed selection in crosses between diverse tomato genotypes and *sldmp* FAST-Red lines. The haploid induction rate (HIR) after crossing 36 different female genotypes with the *sldmp* inducer lines ranged from 0.5% to 3.7%, with an average HIR of 1.9% (Figure 1s). These data suggest that *sldmp* mutants can be used for genotype-independent HI.

To summarize, we demonstrate that *sldmp* mutants induce *in vivo* maternal haploids in a major dicot crop, tomato, and that identification of haploid embryos is facilitated by the FAST-Red marker. Given the presence of *DMP*-like genes in dicot species and the ability of both *Arabidopsis* and tomato *dmp* mutants to induce maternal haploids (Zhong *et al*., 2020), it is highly likely that *dmp* haploid inducers can be generated in other dicot crops. Extending this system would represent a major advance over *in vitro* haploid production, especially for members of the Solanaceae, Fabaceae and Cucurbitaceae that are recalcitrant for DH production (Hooghvorst and Nogués, 2020).

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Conflict of interest

The authors declare no competing interests.

Author contributions

Y.Z., B.C., C.L., K.B. and S.C. conceived and designed the experiments. Y.Z., D.W., B.C. and X.Z. performed most of the experiments. M.L., J.Z., M.C., M.W., T.R., J.L., X.Q., Y.W., D.C., Z.L., J.L., C.C. and Y.J. performed some of the experiments. Y.Z., B.C., S.C., C.L., M.W. and W.L. analysed the data. Y.Z., B.C., S.H., K.B. and S.C. discussed and prepared the manuscript. All authors discussed the results and provided feedback on the manuscript.

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