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***In planta* haploid induction by *kokopelli* mutants**

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Running title: *kokopelli* triggers haploid induction

Keywords: plant breeding, haploid induction, doubled haploid technology, fertilization, pollen, plant reproduction, haploid embryos.

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20 Dear Editors,

21

22 Double haploid (DH) technology is a powerful way to improve plant breeding efficiency (Jacquier et
23 al., 2020). DH breeding based on *in planta* haploid induction represents the most attractive way to
24 induce haploid plants because it leads to seed-based haploid embryo formation, without the need of
25 labor-intensive *in vitro* embryo rescue (Jacquier et al., 2020). This strategy has recently become
26 even more attractive since, in combination with CRISPR-Cas9 technology, it allows gene editing of
27 non-transformable elite lines and the immediate recovery of homozygous mutants (Kelliher et al.,
28 2019). However, the availability of *in planta* haploid induction systems is presently limited to a
29 handful of crop species (Jacquier et al., 2020). In this work, mutation of *KOKOPELLI* (*AtKPL*) was
30 found to be able to triggers maternal haploid induction in *Arabidopsis Thaliana*.

31 The identification of DOMAIN OF UNKNOWN FUNCTION 679 membrane proteins (DMP) as
32 molecular players contributing to *in planta* haploid induction in maize (Zhong et al., 2019), allowed
33 the successful translation of the haploid induction property to Arabidopsis (Zhong et al., 2020) and
34 rapidly to many dicot crops such as tomato, *Brassica napus*, *Nicotiana tabacum*, *Brassica oleracea*
35 and potato (Zhong et al., 2022a; Zhong et al., 2022b; Zhao et al., 2022; Zhang et al., 2022). In
36 addition to producing haploid embryos, knocking-out the two orthologous *DMP* genes in *Arabidopsis*
37 (*Atdmp8/9*) was found to lead to double fertilization defects and seed abortions (Cyprys et al., 2019;
38 Zhong et al., 2020). More precisely, the *Arabidopsis Atdmp8/9* double mutation led to preferential
39 single fertilization of the central cell suggesting a role in the interaction of male and female gametes
40 (Takahashi et al., 2018; Cyprys et al., 2019).

41 In order to identify additional genes exploitable for *in planta* haploid induction, we
42 hypothesized that other mutants impaired in gamete interactions might also induce haploid embryos.
43 We thus searched for genes in the *Arabidopsis* male gametophyte that lead to single fertilization
44 events when mutated, similarly to what had been reported for the *Atdmp8/9* mutant. We selected
45 *AtKPL* (*KOKOPELLI*, *AT5G63720*) gene (Ron et al., 2010; Maruyama et al., 2013) for haploid
46 induction tests. In order to unambiguously detect maternal haploid induction, a female tester line
47 was created that combines both a recessive phenotypic marker, *i.e.* absence of trichomes due to the
48 *glabra1* mutation, and an F1 hybrid genetic background between the Columbia-0 (Col-0) and
49 Landsberg erecta (Ler) accessions (*gl1_Col/gl1_Ler*) (**Figure 1a**). Haploid inducing capacity of *Atkpl*
50 mutants was evaluated by the following pipeline (**Figure 1a**): (1) Mutant pollens were deposited on
51 pistils of the *gl1_Col/gl1_Ler* tester line, (2) the offspring were germinated and screened for absence
52 of trichomes indicating a putative desired maternal haploid seedling, or an undesired self-pollination
53 of the female tester line (contamination) (**Figure 1a**); (3) a first distinction between haploids and
54 contaminations by selfing of the *gl1_Col/gl1_Ler* tester female parent was based on two typical
55 haploid phenotypes: sterility (absence of silique) and smaller organs (**Figure 1a-b**); (4) haploidy was
56 further assessed by flow cytometry (**Figure 1a, c**) and (5) confirmed genetically using 83 single
57 nucleotide polymorphisms (SNPs) markers distributed on all 5 *Arabidopsis thaliana* chromosomes.

58 These 83 SNPs are at a heterozygous state in the female tester line, and are thus expected to
59 behave like homozygous markers in maternal haploid plantlets (**Figure 1a, d**).

60 Two independent mutant T-DNA insertion lines for *AtKPL* were evaluated (*Atkpl-1* and *Atkpl-*
61 *2* in WS-4 and Ler genetic background, respectively) (Ron et al., 2010), and the *Atdmp8/9* double
62 mutant (col0 background) was used as positive control for haploid induction. Using the pipeline
63 described above, the previously reported haploid induction capacity for *Atdmp8/9* double mutant
64 (Zhong et al., 2020) was confirmed since 9/490 plants screened were found to be haploid, *i.e.* a
65 haploid induction rate (HIR) of 1.87% (**Figure 1e**). Interestingly, haploid induction was also observed
66 for both *Atkpl-1* and *Atkpl-2*, with 4 haploids among 1179 plants (HIR 0.34%) and 1 haploid among
67 1389 plant (HIR 0.07%), respectively (**Figure 1e**). Since the three tested mutants (*Atdmp8/9*, *Atkpl-1*
68 and *Atkpl-2*) were in different genetic backgrounds, three different wild-type accessions, Col-0, WS-
69 4 and Ler, were evaluated for their HIR, and no haploid were scored out of 1840, 1738 and 1468
70 plants, respectively (**Figure 1e**). Thus, the HIR of *atkpl-1* is statistically significant in comparison to
71 the HIR of WS-4 wild-type plants (Kruskall-Wallis, p-value = 0.049). All plants classified as haploids
72 passed all steps of the pipeline. The final genetic analyses for haploid plants revealed that (1) all
73 markers were detected as at the “homozygous” state confirming the haploid status of those plants,
74 and (2) 100% of SNPs that could be attributed to a parent came from the maternal parent (**Figure**
75 **1d**). Thus, pollination using *Atkpl-1* and *Atkpl-2* mutant pollen produces true maternal haploid plants.

76 Altogether, these results demonstrate that inactivation of *AtKPL* triggers *in planta* maternal
77 haploid induction and that *Atkpl* mutants could be used as haploid inducer lines. The higher HIR of
78 *Atkpl-1* (0.34%) as compared to *Atkpl-2* (0.07%) might come from difference in allele strength, since
79 Ron et al., (2010) showed *Atkpl-1* has more severed reduced seed set as compared to *Atkpl-2*. The
80 fact that both the *Atdmp8/9* double mutant and *Atkpl* single mutant are impaired in double
81 fertilization, tends to support that single fertilization is an important feature for *in planta* haploid
82 induction. Interestingly, a double mutation in the egg cell specific endopeptidase genes
83 *Atecs1/Atecs2* leads to preferential fertilization of the central cell (Jiang et al., 2022), and is able to
84 confer haploid induction capacity (Mao et al., 2022). Contrary to *Atdmp8/9* and *Atkpl*, *Atecs1/Atecs2*
85 acts from the female side, and thus reinforces the hypothesis that disruption of double fertilization,
86 and probably preferential fertilization of the central cell over the egg cell, is a prerequisite for or a
87 sufficient trigger of haploid induction. It would be interesting to test if combinations of these
88 mutations have additive or synergistic effects on HIR.

89 Although the ~0.34% HIR of *Atkpl-1* is quite low for an application in plant breeding, this may
90 be different in other species. For example, maize *Zmdmp* mutants have a HIR of 0.15% compared
91 to a HIR of ~2% for *dmp* mutants in dicots. In addition, the possible synergy of the *kpl* mutation with
92 other mutations needs to be explored. Indeed, although the maize *Zmdmp* single mutant has a very
93 low HIR (~0.15%), its combination with a mutation in *MATRILINEAL* / *NOT-LIKE-DAD* /
94 *ZmPHOSPHOLIPASE-A1* (*MTL/NLD/ZmPLA1*) leads to a synergistic effect boosting the HIR to 3-4
95 times (Jacquier et al., 2020). Taken together, both the *per se* evaluation of *kokopelli* mutants in other
96 species and the interaction of *kpl* mutation with other mutants drives the interest in this new player in

97 haploid induction. The identification of *AtKPL* orthologs in other species appears straight forward
98 despite the presence of recent duplications in some species, as illustrated by the phylogenetic tree
99 in **Figure 1f**, which depicts some crop plants only. The presence of orthologous genes in both
100 monocotyledonous and dicotyledonous plants can be thus leveraged for setting-up or improving *in*
101 *planta* haploid induction capacity in crops lacking this breeding tool.

102

103

104 **Legend Figure 1**

105 ***Atkpl* triggers maternal haploid plants.**

106 (a) Workflow used to assess maternal haploid induction. Pollen from mutants to be tested were used
107 to pollinate the *glabra* (absence of trichomes) maternal tester line, which is a F1 hybrid between Col-
108 0 and Ler accessions. A first phenotypic screen based on absence of trichome allowed selection of
109 putative haploid plants, which were further validated using sterility criteria, flow cytometry, and
110 genotyping of 83 SNP markers with genome-wide distribution. (b) Representative illustration of
111 diploid plants (left), as compared to the smaller haploid plants (right). (c) Representative ploidy
112 verification of haploid and diploid sibling using flow cytometry for *Atkpl-1*, *Atkpl-2* and *Atdmp8/9*. The
113 X axes depict the DNA content for nuclei (DAPI fluorescence signal), whereas the Y axis represents
114 the number of nuclei. (d) Genotyping results to confirm haploidy and maternal origin of putative
115 haploid plants (sterile *glabra*). m= numbers of markers for which it was possible to track
116 unambiguously their parental origin. 1= Example of diploid plant resulted from undesired selfing of
117 maternal tester line. na= non-applicable. (e) Haploid Induction Rate (HIR). 2= independent crosses
118 are emasculated inflorescence (~1 to 4 siliques) pollinated with a unique male parent. * P<0.05,
119 Kruskal-Wallis statistical test. (f) Phylogenetic tree of KOKOPELLI proteins in selected crops.

120

121 **Supplemental data**

122 The following materials are available in the online version of this article.

123 **Author contributions**

124 **Supplemental Materials and Methods**

125 **Supplemental Table S1.** Sequences of the primers used in this study.

126 **Supplemental Table S2.** KASP markers used in this study to detect SNPs.

127

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140 **Conflict of interest statement**

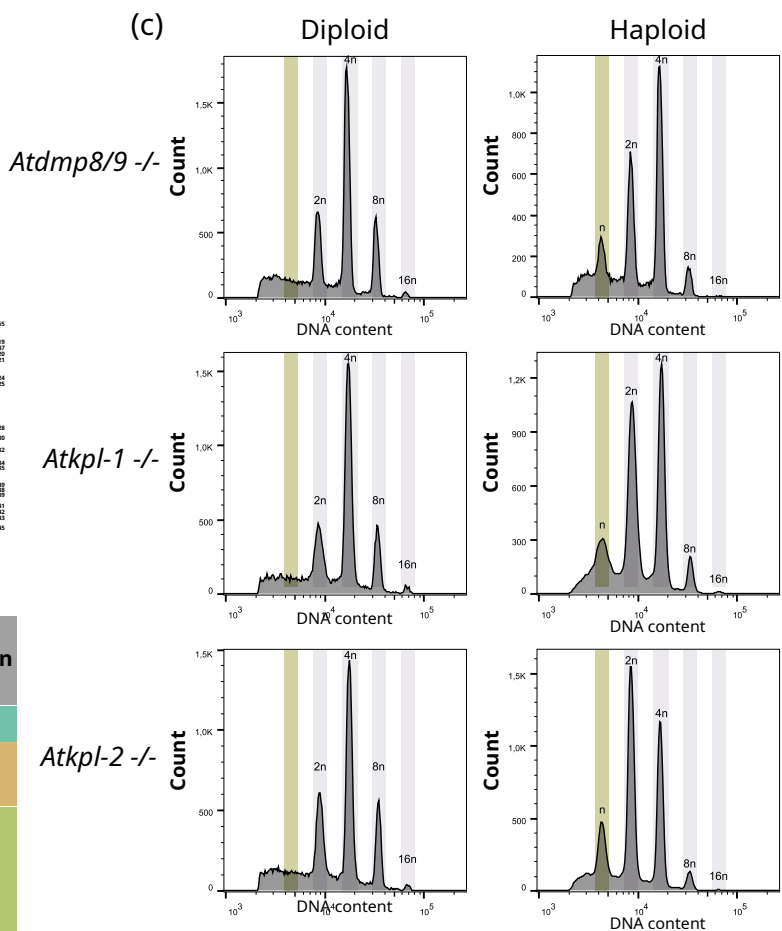
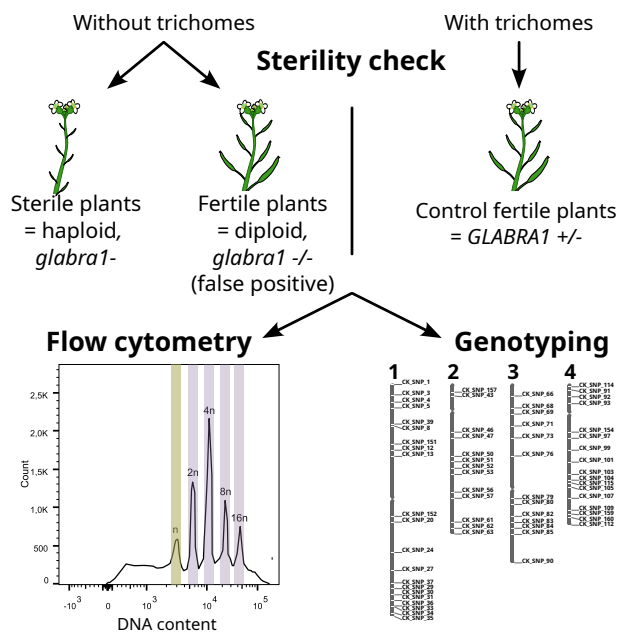
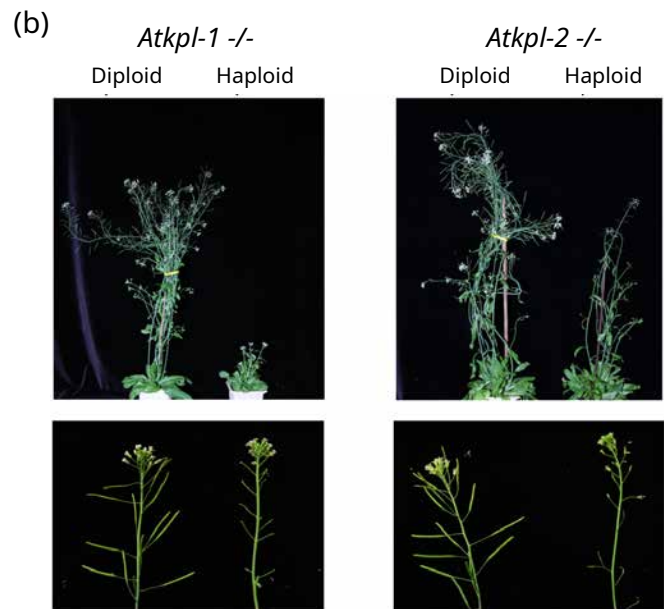
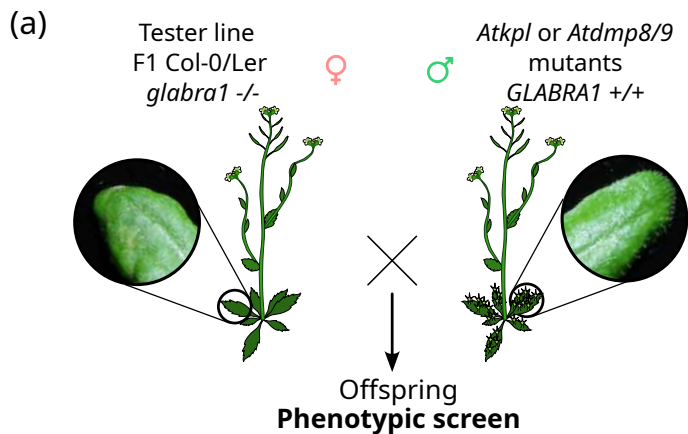
141 A patent application covering the results of this manuscript was submitted. NMAJ, LMG, and JPM
142 are employees of Limagrain Europe. TW and PR are involved in collaborative research with
143 Limagrain Europe. PR is a member of the operational directorate of the PlantAlliance consortium.

144

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175



(d) Summary of heterozygous marker rates and SNP call rates for *Atkpl-1* and *Atdmp8/9* mutants.

Male parent	Plant phenotype	Heterozygous marker rate	Call	SNPs from maternal origin (%)
na	<i>glabra</i> tester line	100%	na	na
<i>Atkpl-2</i>	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=52)
	Fertile trichome	65.4%	Diploid	na
<i>Atkpl-1</i>	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=34)
	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=36)
	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=58)
	Fertile <i>glabra</i>	61.8%	Diploid ¹	na
	Fertile trichome	27.6%	Diploid	na
	Fertile trichome	42.0%	Diploid	na
<i>Atdmp8/9</i>	Fertile trichome	49.7%	Diploid	na
	Fertile trichome	66.3%	Diploid	na
	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=69)
	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=43)
<i>Atdmp8/9</i>	Sterile <i>glabra</i>	0.0%	Haploid	100% (m=42)
	Fertile trichome	62.4%	Diploid	na

(e) Summary of independent crosses and HIR (%) for *Atkpl-1* and *Atdmp8/9* mutants.

Genotype	Accession	Haploid plants	Diploid plants	Total plants	Number of independent crosses ²	HIR (%)
<i>Atkpl-2</i>	Ler	1	1388	1389	n=14	0.07
WT	Ler	0	1468	1468	n=9	0.00
<i>Atkpl-1</i>	WS4	4	1175	1179	n=16	0.34*
WT	WS4	0	1738	1738	n=10	0.00
<i>Atdmp8/9</i>	Col0	9	481	490	n=9	1.87*
WT	Col0	0	1840	1840	n=12	0.00

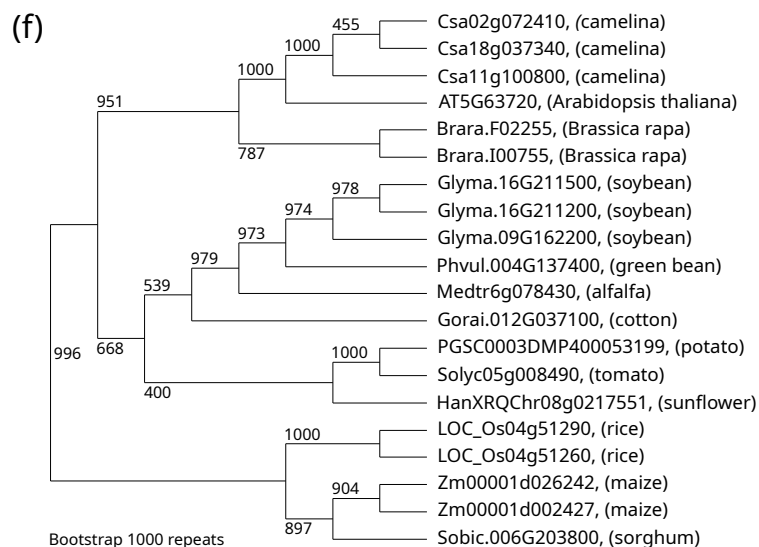


Figure1. *Atkpl* triggers maternal haploid plants.

(a) Workflow used to assess maternal haploid induction. Pollen from mutants to be tested were used to pollinate the *glabra* (absence of trichomes) maternal tester line, which is a F1 hybrid between Col-0 and Ler accessions. A first phenotypic screen based on absence of trichome allowed selection of putative haploid plants, which were further validated using sterility criteria, flow cytometry, and genotyping of 83 SNP markers with genome-wide distribution. (b) Representative illustration of diploid plants (left), as compared to the smaller haploid plants (right). (c) Representative ploidy verification of haploid and diploid sibling using flow cytometry for *Atkpl-1*, *Atkpl-2* and *Atdmp8/9*. The X axes depict the DNA content for nuclei (DAPI fluorescence signal), whereas the Y axis represents the number of nuclei. (d) Genotyping results to confirm haploidy and maternal origin of putative haploid plants (sterile *glabra*). m= numbers of markers for which it was possible to track unambiguously their parental origin. 1= Example of diploid plant resulted from undesired selfing of maternal tester line. na= non-applicable. (e) Haploid Induction Rate (HIR). 2= independent crosses are emasculated inflorescence (~1 to 4 siliques) pollinated with a unique male parent. * P<0.05, Kruskal-Wallis statistical test. (f) Phylogenetic tree of KOKOPELLI proteins in selected crops.

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