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Improving *in vivo* maize doubled haploid production efficiency through early detection of false positives

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With 1 figure and 2 tables

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Abstract

In vivo doubled haploid (DH) technology provides a means of creating new maize inbred lines relatively quickly; however, productivity is limited by false-positive (FP) plants for haploidy and for dihaploidy, which consume resources of space and labour until detected. This work examines the potential for using stomata guard cell length measurement as a means for early detection of FP plants. We found that the true haploid and DH plants could be differentiated from FP and untreated diploid controls as early as Leaf 2 stage by stomata guard cell length measurement. Furthermore, DH plants were distinguishable from haploid and other diploid plants by the Leaf 7 growth stage. Results suggest that, when used together with screening through the anthocyanin colour marker system and flower fertility, stomata guard cell measurement is an easy, non-destructive, early screening method that may lead to a greater efficiency in DH production systems and optimization of resource allocation for space and labour.

Key words: dihaploidy — doubled haploid — quality assurance — maize

In vivo doubled haploid (DH) production is increasingly used in new line development in maize to accelerate the inbreeding process, reduce resource expenditures and increase the precision in testcross evaluation. Doubled haploid production typically involves a number of steps including (i) crossing the 'new line' parents to produce 'donor' materials, (ii) pollinating the resulting F1's or F2's with pollen of the inducer line, (iii) screening the resulting seed for haploids, (iv) germinating haploid seed, (v) applying chromosome doubling agent to seedlings, (vi) self-pollinating DH plants and (vii) recovering seed of new inbred lines. A dominant anthocyanin colour marker system inherent to the inducer line is commonly used as an indicator of ploidy level to identify 1N seeds for further processing (Nanda and Chase 1966, Chase 1969). Haploid seeds display purpling in the endosperm cap but not in the embryo, while 2N seeds display purpling in both endosperm and embryo as well as in the stalk once germinated, and outcrosses display no purpling. However, the expression of the colour markers varies greatly depending on genetic background of the donor and inducer material as well as environmental factors (Chase 1952, Roeber et al. 2005, Kebede et al. 2011, Prigge et al. 2011). For the system to run efficiently, reliable methods are needed to confirm that 1N seeds have been distinguished from diploid seeds resulting from the cross to the inducer and outcrosses (Step 3 above) to minimize false positives (FPs) that needlessly consume greenhouse space, labour and resources.

To further improve the efficiency, it is useful to confirm chromosome doubling of treated haploid plants before flowering (before Step 6 above) to focus labour and resources prior to and at pollination. At present, stalk colour marker expression is commonly used for screening out the FPs in the late seedling stage. Fertility is a prime indicator of DH plants (Eder and Chalyk 2002), while haploid plants remain sterile. However, early non-destructive confirmation methods would be helpful to improve the efficiency of DH production and to optimize the resource allocation.

The length of stomata guard cells in the leaf has been correlated with DNA content in barley, wheat and rye (Borrino and Powell 1988, Limin and Fowler 1989, Singh and Sethi 1995, respectively) and suggested as a possible means to differentiate DH plants from haploid plants in wheat DH production (Sood et al. 2003). Because the chromosome doubling treatment is applied to 3- to 4-day-old seedlings, chimeric tissues can occur within the treated haploid plants. However, because the treatment targets doubling of meristem in order to produce fertile flowers, a distinct increase in DNA content at and beyond a certain growth stage is expected, if the doubling treatment was successful.

In this study, we compared the effectiveness of stomata guard cell length measurement, the anthocyanin colour marker system (through the UH400 inducer) and flow cytometry in detecting FPs at various stages in vegetative growth.

Materials and Methods

Haploids were induced for twelve F1 donor genotypes (Table 1) using inducer UH400 (University of Hohenheim: https://www.uni-hohenheim.de/ipspwww/350a/linien/indexl.html#uh400, Current as of 10 Oct. 2011), which contains the dominant *R1-nj* gene expressing a purple coloration of the scutellum and the aleurone of seeds (Nanda and Chase 1966) and a dominant purple stalk marker (Neuffer et al. 1997). Purple coloration in the endosperm but not the embryo was used as screening criterion to select haploid kernels from the seed bulk per genotype also containing diploid and possibly outcrossed kernels. The inducer line has genetic variation, and the intensity of colour expression varies among individual seeds (W. Schipprack 2010, personal communication). The chromosome doubling treatment was applied on 3- to 4-day-old haploid seedlings (0.06% colchicine with 0.5% DMSO as per Eder and Chalyk 2002); then after 2 weeks,

Table 1: Haploid induction rate, number of haploid seed obtained (N), number of doubled haploid (DH) and rate of chromosome doubling, and false-positive rates (FPR) for screenings by stalk colour and by flow cytometry for each F1 donor genotype

F1 donor genotype	Induction rate (%)	N	DH (chromosome doubling rate %)	FPR by stalk colour (%)	FPR by flow cytometry (%)
AR1705616 × DKXL212	3	48	7 (21.9)	16.7	27.1
B73 × LH1	3	9	4 (57.1)	11.1	33.3
B73 × LH82	4	19	3 (17.6)	36.8	42.1
B73 × PHG35	3	15	2 (14.3)	26.7	53.3
B73 × PHG39	3	22	1 (5.0)	85.7	85.7
B73 × PHG84	3	22	4 (23.5)	54.5	72.7
LH123HT × LH82	3	15	2 (13.3)	73.3	73.3
LH123HT × PHG35	4	13	1 (12.5)	61.5	84.6
LH123HT × PHG39	7	39	0 (0.0)	84.6	84.6
LH123HT × PHG84	2	12	0 (0.0)	54.5	54.5
LH123HT × PHZ51	4	9	0 (0.0)	22.2	22.2
PHG39 × PHG84	3	29	0 (0.0)	51.7	89.3

surviving seedlings were transplanted to the field in Urbana, IL, in the summer of 2011. In addition to the treated haploids, controls included untreated haploids and the untreated inbred parents of the donor F1's. Any presumed haploids that later exhibited 2N status at the earliest leaf stage via flow cytometry were categorized as FPs. Also, plants that exhibited 4N status in flow cytometry (presumably doubled FPs) were excluded from the FP group. Although FP plants were definitively determined using flow cytometry, we also took stalk colour scores on the presumed haploids at one late vegetative stage to check on the rate of FP determined through that method.

DNA content was measured by flow cytometry (following Kim et al. 2010) on individual plants from Leaf 2 through Leaf 10 stage. Stomata guard cell imprints were produced and photographed at 40× magnification to measure the stomata guard cell length (following Kim et al. 2010), with data collected from at least three different leaves (two samples with four measurements each) of all seedlings through the 10th leaf stage. Lastly, male sterility/fertility was observed and recorded as a means to confirm DH plants and distinguish DHs from haploid plants.

Statistical analyses were performed on the stomata guard cell length data using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA, 2003). The analyses were performed using PROC GLM, and the Tukey–Kramer method was used to compare the mean differences.

Results

The haploid induction rates were within the range common for UH400 and relatively similar across donor genotypes (Table 1). Stalk colour marker observations and flow cytometry revealed a high rate of false positives (FPR) (Table 1), with differences across F1 donor genotypes. The reliability of the stalk colour in detecting FPs was assessed through comparison to flow cytometry, which is widely accepted as a dependable method of determining ploidy level (DeLaat et al. 1987). For some genotypes, the FPR was higher with flow cytometry, indicating that stalk colour marker expression may be inhibited by genetic or environmental factors. For five F1 donor genotypes, stalk coloration was as dependable an indicator as flow cytometry in identifying FPs (Table 1); however, no inferences are made about the reliability of the stalk coloration indicator based on specific F1 donor parentage. Overall, based on the F1 donor genotypes in this study, it appears that some FPs slip through haploid screening and may be carried along needlessly in DH production. It should be noted that stalk coloration was not observed on any true haploid plants (i.e. false negatives) in this study.

We found that the true haploid and DH plants could be differentiated from FP and diploid controls as early as Leaf 2 stage by stomata length measurement (Fig. 1). True haploids could be differentiated from all other categories by Leaf 3. The mean stomata length of DH plants was significantly smaller than that of the diploid controls at all leaf stages, possibly due to the occurrence of chimeric tissues. This was consistent with results of flow cytometry (data not shown). Doubled haploid plants were distinguishable from other categories as early as Leaf 5 (Fig. 1). However, the equivalency of FP and diploid controls was not apparent until Leaf 7. Although stomata guard cell length varied significantly among genotypes (Table 2), we found that the relative size differential between haploid, DH and diploid held across genotypes. Thresholds per genotype can be determined prior to DH production by

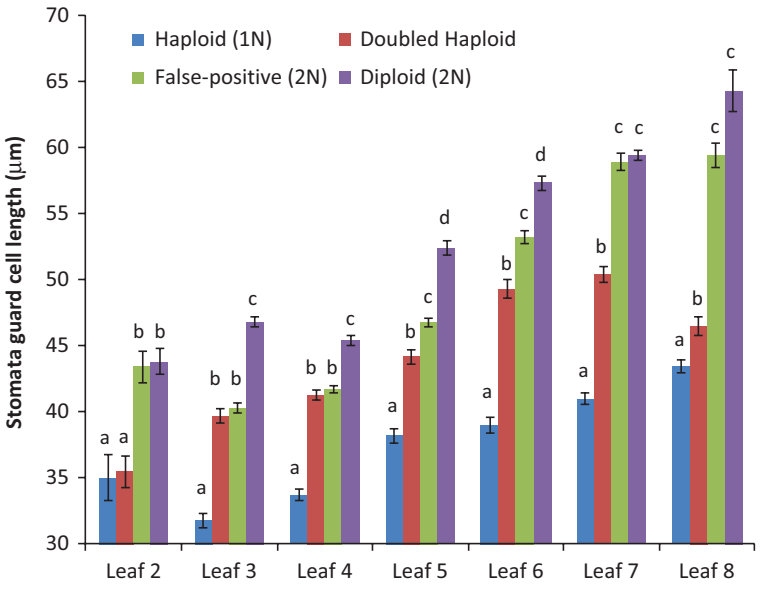

Fig. 1: Mean stomata guard cell length (in µm) in Leaf 2 through Leaf 8 of haploid, doubled haploid (DH), false positives (FP) and diploid across genotypes given with the confidence interval for the mean. Letter categories represent significant differences at the 0.05 probability level

Table 2: Mean stomata guard cell length (in μm) and standard error for various diploid inbreds that served as parents for F1 donor genotypes across Leaf 2 through Leaf 8, showing the variability among the parent lines

Parental genotype	Mean (μm)	Standard error
LH123HT	43.8[a]	0.63
PHG39	45.4[a]	0.65
PHG84	51.9[b]	0.52
PHZ51	52.1[b]	0.59
B73	54.2[bc]	0.53
PHG35	55.9[c]	0.68
LH1	56.5[c]	0.50
LH82	56.7[c]	0.46

Letter categories represent significant differences at the 0.05 probability level.

collecting stomata length data on a small set of donor genotypes and untreated haploid plants at Leaf 3 and Leaf 7 prior to the DH production. These materials establish the upper and lower bounds, with values for DHs expected to be intermediate and statistically distinguishable from both upper (diploid parents) and lower (haploid) means.

Discussion

Overall, stomata guard cell length measurement proved to be an effective means by which to identify haploids and to differentiate between haploid, DH and FP. Together with use of the anthocyanin colour marker system and flower fertility, this measurement could be used to develop Quality Assurance protocols to improve the DH production efficiency and reduce the waste of greenhouse/field space and labour resources. For example, using stomata guard cell measurement at Leaf 7, ~72% of space and labour for growing FP seedlings to pollination and/or maturity would have been saved in this experiment.

Stomata guard cell length measurement can be more efficient than flow cytometry for distinguishing DH from haploid and FP plants. It takes < 5 min per sample and costs about $0.10 per sample to measure stomata guard cell length, while flow cytometry takes about 15 min and costs about $2.00 per sample. Stomata guard cell measurement is also easy and requires only a microscope, slide glasses and super glue to perform, while flow cytometry analysis requires special training and equipment. Moreover, because the presence of chimeric tissues in DH plants can lead to both haploid and diploid peaks in the flow cytometry graph, flow cytometry results require a higher level of expertise for proper evaluation and interpretation.

In conclusion, stomata guard cell length measurement is an easy, low-tech, non-destructive method that shows promise in increasing DH production efficiency by facilitating early identification of FP plants for haploidy and for dihaploidy.

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