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RESEARCH

Development and Validation of Red Root Marker-Based Haploid Inducers in Maize

Vijay Chaikam, Leocadio Martinez, Albrecht E. Melchinger, Wolfgang Schipprack, and Prasanna M. Boddupalli*

ABSTRACT

One of the critical limitations for the in vivo production of doubled haploid (DH) lines in maize (*Zea mays* L.) is the inability to effectively identify haploids in a significant proportion of induction crosses due to the possibility of complete or partial inhibition of the currently used *R1-nj* (Navajo) color marker. In this study, we demonstrate that the *R1-nj* marker could result in a high proportion of false positives among the haploids identified, besides being ineffective in germplasm with natural anthocyanin expression in pericarp tissue. To address these limitations, we developed haploid inducer lines with triple anthocyanin color markers, including the expression of anthocyanin coloration in the seedling roots and leaf sheaths, in addition to the Navajo marker on the seed. Although these inducers show acceptable haploid induction rates ranging from 8.6 to 10.2%, they exhibited relatively poor agronomic performance compared with tropicalized haploid inducers within tropical environments. The addition of the red root marker more accurately identified haploids among the germinating seedlings, including four tropical inbred lines and eight breeding populations that showed complete inhibition of *R1-nj*. We also demonstrate that the red root marker can be used for haploid identification in germplasm with natural anthocyanin expression in the pericarp. A survey of 546 tropical inbreds and 244 landraces showed that anthocyanin accumulation in the roots of germinating seedlings is very rare compared with anthocyanin accumulation in the seed and leaf sheath tissues. As a result, the red root marker can serve as a highly complementary marker to *R1-nj* to enable effective identification of haploids within a wide range of tropical maize germplasm.

V. Chaikam, and L. Martinez, CIMMYT, Apdo. Postal 6-641 06600, Mexico D.F, Mexico; A.E. Melchinger, and W. Schipprack, Institute of Plant Breeding, Seed Science and Population Genetics, Univ. of Hohenheim, D-70593 Stuttgart, Germany; P.M. Boddupalli, CIMMYT, ICRAF campus, UN Avenue, Gigiri, Nairobi, Kenya. Received 26 Oct. 2015. Accepted 24 Dec. 2015. *Corresponding author (b.m.prasanna@cgiar.org).

Abbreviations: DH, doubled haploid; FDR, false discovery rate; FNR, false negative rate; HIR, haploid induction rate; MCC, Matthews correlation coefficient.

The efficient production of inbred lines through the doubled haploid (DH) technology provides significant economic advantages to crop breeding programs (Dunwell, 2010). In maize breeding, use of DH lines enhances genetic gains and improves breeding efficiency via simplification of the logistics and cost reduction for line development and maintenance (Schmidt, 2003; Melchinger et al., 2005; Lee and Tracy, 2009). The DH technology in maize essentially involves in vivo induction using a haploid inducer stock, identification of haploids, doubling the haploid chromosome complement, and the production of seed from fertile DH plants (Prasanna et al., 2012; Prigge and Melchinger, 2012)

In vivo haploid induction is achieved by crossing the source germplasm with pollen from maternal haploid inducers that have the capability to induce haploid embryos. Inducers with 6 to 15% haploid induction rates are now available in both temperate (Röber et al., 2005; Prigge et al., 2012b) and tropical genetic backgrounds (Prigge et al., 2012a). Haploids can be distinguished from diploids based on phenotypic markers or differences in plant characteristics at the adult stage (Xu et al., 2013; Weber, 2014; Wu et al., 2014). However, identification of haploids at the seed or early seedling stage is important for large scale production of

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DH lines, as chromosomal doubling is generally achieved by treating the haploids at the early seedling stage with mitotic inhibitors such as colchicine (Melchinger et al., 2016). The chromosomal doubling procedure is labor- and resource-intensive. Additionally, this technique involves the use expensive and toxic chemicals (Kleiber et al., 2012). Hence, efficient identification of haploids before chromosomal doubling saves considerable resources with respect to the use of chemicals, greenhouse space, field space, labor and expenses related to management of diploids in the field.

The *R1-nj* (Navajo) anthocyanin color marker is widely used for haploid identification and all currently used haploid inducers have the genetic constitution that is necessary for *R1-nj* expression (Melchinger et al., 2015a). *R1-nj* is inherited dominantly and in progeny resulting from the induction crosses, the diploid kernels are marked with purple coloration on the crown region of the endosperm and scutellum of the embryo. In contrast, the haploid kernels show anthocyanin coloration only on the endosperm and no coloration within the scutellum. Thus, the *R1-nj* marker facilitates the visual sorting of haploids from diploids at the seed stage (Nanda and Chase, 1966; Greenblatt and Bock, 1967). However, *R1-nj* fails in haploid identification when (i) *R1-nj* expression is completely inhibited by dominant color inhibitor genes; (ii) *R1-nj* expression is segregating among the kernels of the source germplasm; and (iii) *R1-nj* marker expression is poor with respect to anthocyanin intensity or the marked area. In a set of 897 tropical inbred lines, complete inhibition of *R1-nj* was shown to frequently occur (~30%) and was attributed to the presence of the color inhibitor *C1-I* (Chaikam et al., 2015). In addition, ~70% of the 155 landraces and ~40% of 157 breeding populations showed segregation for *R1-nj* expression. Hence, the use of *R1-nj* is not efficient in such germplasm (Chaikam et al., 2015). Even when expressed, poor intensity of the *R1-nj* marker expression can result in high rates of misclassification in temperate flint germplasm (Röber et al., 2005; Melchinger et al., 2014) and tropical landraces (Prigge et al., 2011). Physiological factors such as high moisture content (Rotarenco et al., 2010) and the development of air pockets underneath the pericarp (Prigge et al., 2011) can also affect the efficiency and accuracy of *R1-nj* based haploid identification. Another potential problem is the masking of *R1-nj* phenotype by natural anthocyanin coloration in the seed, especially in the pericarp, of maize landraces.

Two non-anthocyanin based alternatives were proposed for haploid identification to address the above-mentioned limitations of the *R1-nj* marker. Pollination with haploid inducers with sufficiently high kernel oil content, followed by rapid differentiation of haploid kernels based on their lower oil content using Nuclear Magnetic Resonance Spectroscopy (NMR), has been previously demonstrated (Melchinger et al., 2013, 2015b). This method facilitates high-throughput identification of haploids through automation. However, high oil haploid inducers and automation technology are not yet widely available and could be relatively expensive. Transgenic herbicide tolerance was shown to aid in the unambiguous identification of haploids from diploids in genotype-independent manner (Geiger et al., 1994). However, this method is labor-intensive and cannot be employed at the seed or germinating seedling stage (Röber et al., 2005); in addition, the approval process for genetically modified organisms may limit or prohibit its use in many countries.

Two additional anthocyanin marker systems based on stem and root coloration have been previously proposed (Röber et al., 2005; Li et al., 2009; Rotarenco et al., 2010) to complement *R1-nj* based haploid identification. A purple or red stem and sheath color marker conditioned by the anthocyanin regulatory gene *Pl* was integrated into some inducer lines such as RWS (Röber et al., 2005), UH400 (Univ. of Hohenheim, https://plant-breeding.uni-hohenheim.de/84531#jfmulticontent_c167370-2) and CAUHOI (Li et al., 2009). Use of these inducers for induction crosses results in diploids with purple or red stems and sheaths and haploids with green stems and sheaths, provided the source germplasm does not express the *Purple* (*PI*) gene itself. However, purple coloration in the stem and sheath is only expressed at the late seedling or adult plant stage (Röber et al., 2005). On the contrary, the red root marker can be expressed in the roots of germinating seedlings. Rotarenco et al. (2010) demonstrated the use of purple or red root phenotype for distinguishing haploids from diploids at an early seedling stage in a population that completely inhibited *R1-nj* expression. Thus, this marker could effectively complement *R1-nj* in visual identification of haploids at early seedling stages. However, it was not known whether the red root marker is effective within a wide range of tropical maize germplasm for haploid identification as anthocyanin expression in roots could be affected by the genetic background of the source germplasm. In addition, the frequency of natural occurrence of anthocyanin coloration in roots of maize germplasm is unknown. Natural anthocyanin expression in seedling roots could potentially mask the root marker expression in the progeny resulting from an induction cross between source germplasm and the inducer having the genetic constitution for red root marker expression.

In this study, we present newly developed haploid inducer lines that possess three anthocyanin markers; namely *R1-nj*, purple sheath and stem, and red root. Due to the combination of the multiple marker traits, we termed these as "triple marker inducer lines". The objectives of this study are to: (i) determine the accuracy of haploid and diploid seed classification based on the *R1-nj* marker system in terms of false discovery rates and

false negative rates; (ii) evaluate haploid induction rates and agronomic traits of triple marker inducer lines; (iii) validate the usefulness of the red root marker in haploid identification when the *R1-nj* marker is either completely inhibited or masked; and (iv) analyze the prevalence of natural anthocyanin expression within different plant tissues of tropical maize germplasm.

MATERIALS AND METHODS

Testing the Accuracy of Haploid and Diploid Classification with *R1-nj*

The tropicalized haploid inducer hybrid TAIL9 × TAIL8 (http://www.cimmyt.org/es/que-hacemos/investigacion-sobre-maiz/item/tropicalized-maize-haploid-inducers-for-doubled-haploid-based-breeding) was crossed to nine diverse breeding populations and two synthetic populations obtained from different breeding programs in CIMMYT during the winter cycle of 2015 at the CIMMYT Agua Fria experimental station located in the state of Puebla in Mexico (20.26° N, 97.38° W). Seed resulting from the crosses were visually classified into putative haploids and diploids based on the Navajo phenotype, as previously described (Chaikam and Prasanna, 2012). For confirming the true ploidy status, 500 seeds from each of the putative haploid and putative diploid fractions were planted from each population in the field and the surviving plants were evaluated at anthesis stage using a "gold standard" classification based on visual assessment of differences in plant vigor, erectness of leaves, and male fertility in haploids and diploids. Seeds that failed to germinate and the plants that did not survive till anthesis were not taken into account. Putative haploids and putative diploids that were confirmed to be true were categorized as true positives (TP) and true negatives (TN), respectively. True diploids in the putative haploid fraction and true haploids in the putative diploid fraction were categorized as false positives (FP) and false negatives (FN), respectively. The numbers of TP, TN, FP, and FN were adjusted in each population based on the total number of putative haploids and diploids identified within the population based on the *R1-nj* marker. The resulting data from the "gold standard" classification was used to calculate the statistics of false discovery rate (FDR), false negative rate (FNR), and Matthews correlation coefficient (MCC) (Matthews, 1975; Altman and Bland, 1994a, 1994b; Melchinger et al., 2013) according to the following formulae:

$$\mathrm{FDR} = \frac{\mathrm{FP}}{\mathrm{TP} + \mathrm{FP}}$$

$$\mathrm{FNR} = \frac{\mathrm{FN}}{\mathrm{TP} + \mathrm{FN}}$$

$$\mathrm{MCC} = \frac{(\mathrm{TP} \times \mathrm{TN}) - (\mathrm{FP} \times \mathrm{FN})}{\sqrt{(\mathrm{TP} + \mathrm{FP})(\mathrm{TP} + \mathrm{FN})(\mathrm{TN} + \mathrm{FP})(\mathrm{TN} + \mathrm{FN})}}$$

where FDR is the proportion of diploids in the seed fraction classified as putative haploids, FNR is the proportion of true haploids misclassified as diploid seed, and MCC indicates the correlation between the real and predicted ploidy status based on the *R1-nj* marker. MCC has been suggested as a balanced measure of quality of binary classifications especially when the binary classes are of very different sizes (Matthews, 1975). As the numbers of haploids are significantly less than the number of diploids in this study, MCC is preferred over other statistical parameters like the *F*-score.

Development of Triple Marker Haploid-Inducer Lines

Two temperate haploid inducers (UH400 and RWS) from the University of Hohenheim, Germany, were used as sources for haploid induction ability, in addition to the Navajo marker expression and purple stem coloration carried by these inducers. The red root phenotype was identified in population ([Pool16 × ZAPACHI] × Pool16) × ZAPACHI obtained from the CIMMYT maize germplasm bank. This population was crossed to a temperate haploid inducer hybrid RWS × UH400. The F_1 was backcrossed to RWS × UH400, and the best agronomic plants were selected in the subsequent three backcross generations followed by ear-to-row pedigree breeding. A set of 71 S_4 lines was selected and evaluated for the red root phenotype, and 21 lines that expressed the red root phenotype were self-pollinated for two additional cycles. During this process, selections were made within each line for intense red color expression in the roots and good agronomic characters (plant vigor, standability, tassel size, and ear quality). These lines were evaluated for haploid induction rate (HIR) in the summer cycles of 2012 and 2013 at the CIMMYT El Batan experimental station in Mexico (19.52° N, 98.88° W) by crossing the candidate inducers to a *liguleless* tester (homozygous for the *lg2* gene) and scoring for the liguleless phenotype on testcross seedlings as described by Prigge et al. (2012a). Ten candidate haploid inducers with more than 6% HIR were further evaluated in the 2014 winter cycle at Agua Fria, 2014 summer cycle at Metztitlan, state of Hidalgo, Mexico (20.6° N, 98.76° W) and 2015 winter cycle at Agua Fria to determine the stability of HIR and plant characteristics. Agronomic characteristics of 10 candidate inducers were evaluated alongside the tropical and temperate inducers in the 2015 winter cycle at the Agua Fria experimental station. Plant height was measured from the soil surface to the base of the flag leaf. Ear height was measured from the soil surface to the upper ear attachment for ten plants within the middle of the row. Plant aspect was scored on a scale of 1 to 5, where 1 = strong, healthy plants and 5 = very poor, weak and disease-infected plants. Days to anthesis was recorded as the number of days from sowing to the time when pollen shedding occurred in more than half of the individuals within an inducer line. The root and leaf sheath colors were scored on a scale of 1 to 5 where 1 = deep purple or red coloration and 5 = no coloration.

Validation of Red Root Marker Expression in Haploid Identification

To validate the effectiveness of the red root marker, four diverse CIMMYT maize lines (CML376 from the subtropical breeding program in Mexico; CML494 from the lowland tropical breeding program in Mexico; CML506 and CML508 from the subtropical breeding program at CIMMYT-Zimbabwe) and eight lowland tropical CIMMYT-Mexico maize breeding populations, that showed complete inhibition of the *R1-nj* marker (Chaikam et al., 2015) were grown in the 2014 summer cycle at Metztitlan and pollinated with the bulk of pollen

from the triple marker inducers CMRRI003, CMRRI005, CMRRI009 and CMRRI010. After harvest, ears were visually assessed for *R1-nj* expression. Seed were germinated on paper towels for 72 h in a growth chamber and maintained at 28 to 30°C under dark conditions as previously described in Chaikam and Mahuku (2012). Subsequently, the seedlings were maintained in a cold room at 8 to 12°C in the dark for 24 h to suspend growth and allow the accumulation of anthocyanin. Seedlings were separated into putative haploids and putative diploids based on white and red root phenotype, respectively, and were transplanted to the field. Haploids and diploids were reconfirmed in the field based on the "gold standard" classification, that is, a visual assessment of differences in plant vigor, erectness of leaves and male fertility in haploids and diploids. The statistics FDR, FNR and MCC were calculated for the *R1-nj* classification. To validate the use of the *R1-nj* and red root markers in germplasm containing natural expression of anthocyanin in the seed, two blue and one red corn synthetic maize varieties were obtained from the CIMMYT highland maize breeding program in Mexico. These synthetics were grown in the 2014 summer cycle at Metztitlan, Mexico and were crossed with bulked pollen from the triple marker inducers CMRRI003, CMRRI005, CMRRI009 and CMRRI010. *R1-nj* expression in the progeny from these induction crosses was visually determined in the seed at physiological maturity. To validate the use of red root marker for haploid identification, seeds from these induction crosses were germinated and seedlings were separated into putative haploids and putative diploids based on root color. True ploidy was assessed using the "gold standard" classification (per adult plant characteristics) and the associated statistics were calculated as described above.

Natural Expression of Anthocyanin in Different Maize Tissues

Seed for 546 CIMMYT lines and 244 landrace accessions were obtained from the CIMMYT maize germplasm bank. Fifty seeds from each entry were visually assessed for the presence of anthocyanin coloration and the Navajo phenotype in the endosperm and embryo. If a purple or red pericarp was detected, the seeds were broken to determine if anthocyanin was accumulated only in the pericarp or also in the endosperm and embryo tissues; this is the identifying expression characteristic of the Navajo marker.

To determine the presence of anthocyanin coloration in the roots, 25 seeds of each CIMMYT line and landrace accession were germinated in two replications on paper towels along with the triple marker inducer controls as described above. The paper towels were spread under room light and germinating seedlings were assessed for the expression of anthocyanin in the primary and secondary roots. Anthocyanin coloration in the leaf sheath was scored at anthesis in 10 plants grown in the field. For determining the expression in the stem, leaf sheaths below the ear nodes were removed and anthocyanin accumulation was scored.

RESULTS

Accuracy of Haploid Identification Based on *R1-nj* Expression

Statistics associated with binary classification for true ploidy test using the "gold standard" classification for a portion of putative haploids and putative diploids identified in eleven populations based on *R1-nj* were presented in Table 1. FDR ranged from 1.8 to 43.8% among the 11 populations; the overall FDR value was 24.2%. The majority of the populations studied (8 of 11) had FDR values exceeding 20%. FNR ranged from 0 to 39.5% in the 11 populations; overall FNR across all populations was 8.9%. The MCC value ranged from 0.65 to 0.96 among the populations and averaged 0.82 overall. The statistics indicate the possibility of higher misclassification rates when the *R1-nj* marker is used for haploid or diploid classification in tropical germplasm.

Characteristics of the Triple Marker Haploid Inducer Lines

The triple marker inducers show expression of the Navajo phenotype in the seed, red coloration in the roots under darkness and purple or red coloration in the stem (Fig. 1). Both primary and secondary roots develop anthocyanin coloration in darkness from 72 h after germination. Seedlings have white hypocotyls with green tips. In adult plants, stems have intense purple coloration. Although leaf sheaths develop intense purple coloration, leaf blades and margins do not show any anthocyanin coloration. Tassel branches are green in color with purple anthers, which

Table 1. Statistics associated with the "gold standard" classification for confirming the true ploidy of putative haploids and putative diploids identified using the *R1-nj* marker in induction crosses generated with the tropicalized haploid inducer hybrid TAIL9 × TAIL8.

Source germplasm	*n*†	FDR‡	FNR§	MCC¶
		%		
G15 C34 HS#63-1-1-2-2-1-1/CLWN201-B	869	32.0	0.0	0.82
DTPWC9-F67-2-2-1-B/SM-189-79-B	609	7.0	39.5	0.75
CML341/CML440-B	707	24.6	10.0	0.82
Population AZ1	792	43.8	22.3	0.65
Population P4	749	27.6	0.0	0.85
Syntaadenthct1	777	31.0	12.8	0.77
Syntbbflintcomp1	820	24.4	12.0	0.81
CLYN460/CLYN466	738	4.2	13.9	0.90
CLYN453/CLYN489	851	1.8	6.5	0.96
CML511/CML383	829	34.8	0.0	0.80
CML377/CML383	839	32.2	7.0	0.78
Overall	8580	24.2	8.9	0.82

† *n* = total number of plants evaluated in the "gold standard" classification.

‡ FDR = false discovery rate.

§ FNR = false negative rate.

¶ MCC = Matthews correlation coefficient.

Fig. 1. Phenotypic characteristics of triple marker inducer lines. Root coloration in TAIL9 (left) and triple marker inducers (right) after 72 (a), 84 (b), and 96 (c) hours after germination. Adult plant (d), tassel (e) ear (f) and seed phenotypes (g) are also shown.

become yellowish purple when dry. The Navajo phenotype expression on the seed varies among these haploid inducers, with some showing red pigmentation throughout the endosperm and embryo; while in others, the Navajo marker is bluish purple and specifically expressed on the crown and scutellum area.

Table 2 lists the haploid induction rates (HIR) and associated 95% confidence intervals for the triple marker inducer lines in comparison with selected temperate and tropical inducers based on the results obtained with a *liguleless* tester. HIR varied from 8.6 to 10.2% among the four triple marker inducers. CMRRI010 showed the highest induction rate of 10.2% among the triple marker inducers. Two temperate inducers showed average HIR >10% and two tropical inducers showed HIR of 7.5 and 9.9%, respectively. Table 3 presents the agronomic characteristics of the triple marker inducers in comparison with the parental temperate inducer lines and tropical inducer lines. Plant height, ear height and plant aspect at flowering for triple marker inducers were better than the temperate inbred RWS but poorer than in UH400. All of the triple marker inducers showed poorer performance for plant traits than the tropical inducer lines. Among the four triple marker inducers, CMRRI010 showed relatively better agronomic performance. With the exception of CMRRI010, all three inducers showed similar anthesis dates as the temperate inducers. CMRRI010 showed a slight flowering delay in relative compared to other triple marker inducers. All triple marker inducers showed intense red coloration in the root when grown under dark conditions. On the other hand, the temperate and tropical inducers did not show any root pigmentation under the same conditions. CMRRI005 and CMRRI010 showed a relatively higher intensity of anthocyanin coloration in the roots as compared to other triple marker inducers. All of the triple marker inducers showed more intense purple coloration in the leaf sheath compared to the tropical or temperate inducers.

Table 2. Means, 95% confidence intervals, and ranges associated with haploid induction rates (HIR) of triple marker inducers in comparison with tropical and temperate inducer lines assessed based on a *liguleless* tester in the winter cycle of 2014 at the CIMMYT Agua Fria experimental station, Mexico.

Inducer	No. of testcross seedlings evaluated	HIR mean	HIR confidence interval	HIR range
CMRRI003	921	9.7	7.5–11.8	7.5–13.5
CMRRI005	851	8.7	7.6–9.8	7.3–10.5
CMRRI009	920	8.6	7.3–9.9	6.6–10.4
CMRRI010	865	10.2	9.0–11.3	8.7–12.0
RWS	920	13.2	11.7–14.6	11.3–15.8
UH400	951	10.3	7.9–12.7	8.0–14.5
TAIL8	901	9.9	7.9–11.9	7.3–12.5
TAIL9	935	7.5	5.5–9.6	5.6–11.4

Table 3. Agronomic characteristics plant height, ear height, plant aspect at flowering, days to anthesis, and the intensity of anthocyanin expression in roots (IAR) and leaf sheaths (IAS) among triple marker, tropical and temperate inducer lines.

Inducer	Plant height	Ear height	Plant aspect†	Days to anthesis	IAR‡	IAS‡
	cm		"1–5"	d	"1–5"	
CMRRI003	65.1	17.7	3.0	63	2.0	1.0
CMRRI005	60.9	18.4	3.5	71	1.0	1.0
CMRRI009	52.2	19.1	3.0	73	3.5	1.0
CMRRI010	70.1	14.5	2.5	75	1.5	1.0
RWS	58.0	17.8	3.5	70	5.0	4.0
UH400	84.1	26.3	3.0	73	5.0	3.0
TAIL 8	114.6	35.9	2.5	77	5.0	4.0
TAIL 9	97.8	39.6	2.0	76	5.0	3.5

† Plant aspect at flowering was scored on a scale of 1 to 5, where 1 = strong, healthy plants and 5 = very poor, weak and disease-infected plants.

‡ Intensity of anthocyanin expression in roots and leaf sheaths was scored on a scale of 1 to 5, where 1 = deep purple or red coloration and 5 = no coloration.

Validation of the Red Root Marker in Crosses with Complete Inhibition of the *R1-nj* Marker

When crossed with pollen from the triple marker inducer lines, four CIMMYT maize lines and six populations did not show Navajo marker expression on the seed (data not shown). As expected, the majority of germinating seedlings expressed purple or red coloration in the roots while few seedlings showed no root coloration. When the seedlings showing purple or red and white roots were grown separately in the field till anthesis, the majority of the purple or red root plants showed good vigor, broad and erect leaves and good pollen fertility. All of these seedlings were confirmed to be diploids. The majority of plants with white roots at the seedling stage showed poor vigor, narrow and erect leaves with no or poor male fertility. Seedlings with these characteristics were confirmed to be haploids.

The statistical parameters for the binary classification of diploids and haploids, based on the "gold standard" classification for putative haploids and diploids with root coloration, are presented in Table 4. False discovery rate ranged from 0 to 55.6% and the overall FDR remained at 13.9%. The majority of the germplasm studied (9 of 12) had FDR values smaller than 15%. False negative rate ranged from 0 to 16.7% across populations and the overall FNR remained at 6.1%. The MCC value ranged from 0.61 to 0.98 and the overall MCC value averaged 0.89. Thus, our study clearly demonstrates that, on the basis of root coloration, one can reliably differentiate haploids from diploids in crosses with the triple marker inducers, even when the induction crosses show complete inhibition of the Navajo kernel color phenotype.

Table 4. Statistics associated with the "gold standard" classification for confirming true ploidy of putative haploids and putative diploids identified using the red root marker in induction crosses with complete inhibition of *R1-nj*. Bulked pollen from triple marker inducers, CMRRI003, CMRRI005, CMRRI009 and CMRRI0010 was used for induction crosses.

Source germplasm	*n*†	FDR‡	FNR§	MCC¶
		%		
CML376	438	13.5	2.2	0.91
CML494	499	55.6	9.1	0.61
CML506	425	23.3	4.2	0.84
CML508	440	9.5	9.5	0.90
F_1–341	521	8.1	2.5	0.94
F_1–363	532	3.4	12.5	0.91
F_1–364	432	4.4	0.0	0.98
F_1–316	428	0.0	8.0	0.95
F_1–317	418	6.3	16.7	0.87
(CLWN201/CLWN244)-B	426	14.3	7.7	0.88
(CLWN201/CLWN247)-B	416	9.3	0.0	0.95
(CLRCY017/CLYN352)-B	409	15.7	6.5	0.87
Overall	5384	13.9	6.1	0.89

† *n* = total number of plants evaluated in the "gold standard" classification.

‡ FDR = false discovery rate.

§ FNR = false negative rate.

¶ MCC = Matthews correlation coefficient.

Validation of the Red Root Marker in Crosses with Masking of the *R1-nj* Marker Expression

The seed of blue and red corn synthetics used in this study showed deep intense purple and red coloration, respectively, in the pericarp covering the entire seed. When crossed with pollen from the triple marker inducers, *R1-nj* expression is completely masked by anthocyanin expression in the pericarp (Fig. 2). Consequently, it was not possible to separate haploids and diploids based on *R1-nj* expression alone. When the seeds were germinated, the majority of the seedlings showed red coloration in the root tissues while few seedlings showed white roots. When the red and white root seedlings were grown separately in the field till anthesis, the majority of purple-root plants were confirmed to be true diploids and the majority of white-root plants as true haploids (Table 5). The FDR for two blue corn synthetics ranged from 27.2 to 35%, while FDR was 13% for the red corn synthetic. The FNR was lower for the blue corn synthetics, which ranged from 1.5 to 3.7% as compared to 9.1% for the red corn synthetic. The MCC for the blue corn synthetic 1 and 2 was 0.82 and 0.78, respectively, whereas MCC was 0.88 for the red corn synthetic. Taken together, these statistics indicate that the red root marker can aid in identification of haploids in germplasm that masks the expression of the Navajo phenotype.

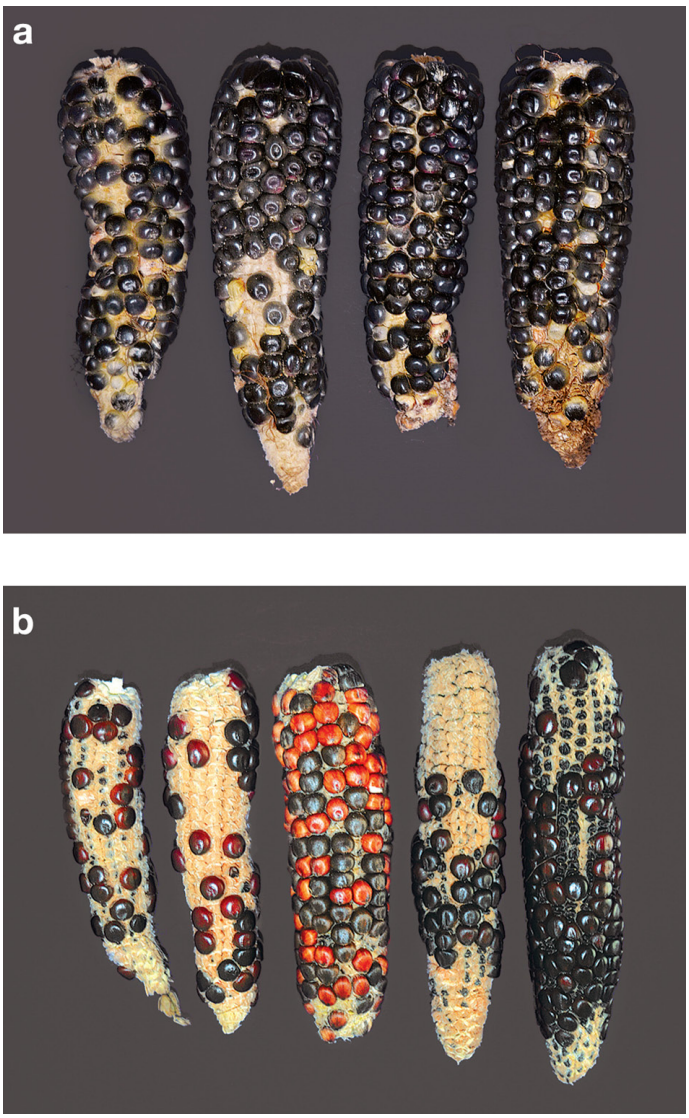

Fig. 2. Masking of *R1-nj* (Navajo) expression in (a) blue corn synthetic 1, and (b) red corn synthetic 1 crossed with triple marker inducers.

Frequency of Natural Anthocyanin Expression in Different Maize Tissues

The frequency of natural expression of anthocyanin in different plant tissues of tropical maize germplam is presented in Table 6. Anthocyanin coloration is not present in the endosperm and embryo of elite maize inbreds developed at CIMMYT (with the exception of some blue maize lines/hybrids developed at CIMMYT-Mexico). On the other hand, in the seed of maize landrace accessions, anthocyanin expression occurs quite frequently (40.9%). However, of the 100 maize landraces that express anthocyanin coloration in the seed, 55 showed segregation for the presence/absence of anthocyanin pigments (data not shown). Among the landrace accessions that express anthocyanins in the seed, only two accessions (Morelia 43 and Veracruz 56) showed Navajo marker expression. None of the elite maize inbreds (CIMMYT lines) showed root coloration under dark conditions, whereas 21 landraces (8.6%) (Supplemental Table S1) showed anthocyanin accumulation in root tissue. All of these accessions showed segregation among seedlings for presence or absence of anthocyanin coloration in the root. For accessions with expression of the red root phenotype, the frequency of red root occurrence (i.e., the proportion of plants with red roots in an accession among the total number of plants germinated in that accession) varied from 4 to 40% (average 17.5%) (data not shown). With regard to the leaf sheath coloration, 9.8% of the inbred lines showed purple coloration, while 60.2% of the landrace accessions revealed sheath coloration. The majority (91.8%) of the accessions showing purple coloration in the sheath also displayed

Table 5. Statistics associated with "gold standard" classification for confirming the true ploidy of putative haploids and putative diploids identified using the red root marker in induction crosses with blue and red corn synthetics. Bulked pollen from the triple marker inducers, CMRRI003, CMRRI005, CMRRI009, and CMRRI0010 was used for the induction crosses.

Source germplasm	*n*†	FDR‡	FNR§	MCC¶
		%		
Blue corn synthetic 1	567	27.2	1.5	0.82
Blue corn synthetic 2	533	35.0	3.7	0.78
Red corn synthetic 1	391	13.0	9.1	0.88
Overall	1491	27.1	3.4	0.82

† *n* = total number of plants evaluated in the "gold standard" classification.

‡ FDR = false discovery rate.

§ FNR = false negative rate.

¶ MCC = Matthews correlation coefficient.

Table 6. Frequency of natural anthocyanin expression in different tissues of tropical maize inbreds and landraces.

Germplasm type	n†	Anthocyanin coloration in seed	Navajo phenotype in seed	Anthocyanin coloration in root	Anthocyanin coloration in leaf sheath	Anthocyanin coloration in stem
		% of germplasm evaluated				
Tropical inbreds vs. landraces						
Tropical inbreds	546	0	0	0	9.8	0.4
Landraces	244	40.9	0.8	8.6	60.2	13.1
Tropical inbreds by adaptation						
Highland CMLs	32	0	0	0	9.4	0
Subtropical CMLs	201	0	0	0	9.9	0
Lowland tropical CMLs	313	0	0	0	9.9	0.6
Landrace accessions by adaptation						
Tropical accessions	151	39	0.6	8.6	53.6	15.8
Subtropical accessions	52	42.3	1.9	1.9	63.4	5.8
Highland accessions	41	46.3	0	17.0	80.4	12.2

† *n* = number of entries evaluated.

segregation (presence/absence) of sheath color. While only two inbreds showed coloration in the stem, 13.1% of the landrace accessions expressed anthocyanin coloration in the stem; of these, 90.6% showed segregation for stem color expression. In the elite inbreds, the frequency of anthocyanin expression in the leaf sheath and stem was similar. Among the landraces, highland accessions showed red root and purple leaf sheath phenotype more frequently than subtropical and lowland tropical accessions.

DISCUSSION

Doubled haploid lines are commonly produced from biparental populations generated from elite maize inbred lines (Prigge et al., 2011). These DH lines can also be produced from landraces and open-pollinated varieties for purification and fixation, as well as for enhancing the genetic diversity of maize breeding programs (Wilde et al., 2010; Strigens et al., 2013). However, inability to identify haploids because of complete or partial inhibition of the currently used *R1-nj* (Navajo) anthocyanin marker is a critical limitation for DH-line production from many breeding populations and landrace accessions (Chaikam et al., 2015). In addition, it has been previously noted that use of *R1-nj* has resulted in a high number of false positives (true diploids among putative haploids) (Röber et al., 2005; Prigge et al., 2011; Choe et al., 2012; Melchinger et al., 2014). It is also possible that all haploids present in the induction cross could not be detected based on *R1-nj* marker expression and can also be falsely included among the diploids (Melchinger et al., 2015b). Röber et al. (2005) showed that temperate flint germplasm with high FDR also have high FNR. To date, no publications which demonstrated the determination of FNR from *R1-nj* based on induction crosses in tropical germplasm. The possible occurrence of high FNR in haploid/diploid classification based on the Navajo phenotype may necessitate the planting of additional plants from the source germplasm to ensure that a sufficient number of haploids can be obtained. In this study, we systematically evaluated both FDR and FNR resulting from *R1-nj* based classification within induction crosses of 11 tropical populations. This study revealed that overall, *R1-nj* haploid/diploid classification can result in FDR >24% in the analyzed tropical maize germplasm. FDR depended on the source germplasm, with some germplasm showing very high values (>40%). Higher FDR of *R1-nj* based haploid/diploid classification leads to wastage of resources during chromosomal doubling and the generation of seed for DH lines. In the majority of the populations used in this study, relatively few true haploids were falsely identified as diploids using the *R1-nj* marker, resulting in an overall low FNR (8.9%). However, similar to FDR, FNR is also dependent on the source germplasm with two populations having >20% FNR. In populations with high FNR, a significant proportion of true haploids will be misclassified in the diploid fraction. A high FNR results in a failure to identify all of the haploids present in the induction cross. As a result, fewer haploids are available for downstream chromosomal doubling. Consequently, this may result in fewer DH lines from a population than desired. A high frequency of *R1-nj* marker inhibition, taken together with high levels of FDR in most of the germplasm and high levels of FNR in some tropical maize germplasm, necessitates alternative or complementary haploid identification marker systems that can effectively address these limitations.

The triple marker inducers developed in this study express the *R1-nj* based Navajo phenotype in seeds, the purple color marker in both the primary and secondary roots, and purple coloration in leaf sheaths and stems. Unlike the temperate and tropical inducers used in this study that do not show root color phenotype under experimental conditions, the triple marker inducers have the genetic constitution required for anthocyanin accumulation in seedling roots. Moreover, these inducers showed reasonably good haploid induction rates for use in haploid induction nurseries. However, generally speaking, the agronomic performance of the triple marker inducers

in terms of plant height, ear height and plant aspect at flowering, was relatively poor as compared to the first-generation tropical inducers (TAIL8 and TAIL9) that were jointly developed by CIMMYT and the University of Hohenheim, Germany. It is possible that the poor agronomic performance of the triple marker inducers could be the result of using landrace accessions and temperate inducers as parents, and the testing of the inducers in tropical environments. In a previous study, temperate haploid inducers showed poor agronomic performance in the tropical environments (Prigge et al., 2012a).

Triple marker inducer lines were used to validate the usefulness of the red root marker for identifying haploids from germplasm that completely inhibits the expression of the Navajo kernel phenotype, conditioned by *R1-nj*. When 12 diverse tropical germplasm were crossed with pollen from the triple marker inducers, all crosses showed complete inhibition of Navajo marker expression but showed segregation for purple coloration in the roots. These observations indicated that the color inhibitor genes do not influence the anthocyanin accumulation in the seedling root tissues. The haploid/diploid classification based on the red root marker in these 12 crosses established that it could be a potential alternative for haploid identification when *R1-nj* expression is inhibited. The FDR was less than 10% in 75% of the germplasm used in this experiment. This observation was significant considering that only 28% of the germplasm showed an FDR less than 10% when using the *R1-nj* marker. However, similar to the *R1-nj* marker, the red root marker also resulted in an exceptionally high FDR values in some source germplasm such as inbreds CML494 and CML506. When using the red root marker, higher FDR in some germplasm can result from slow germination and growth of some diploids due to infection with ear rot fungi, which delays the accumulation of anthocyanins in the roots. In addition, in some germplasm, the poor intensity of anthocyanin coloration in roots at the seedling stage could lead to human errors and a higher FDR. Lower FNR values were noticed using the red root marker as compared to the *R1-nj* marker (overall 6.1 vs. 8.9%), indicating that relatively lower numbers of true haploids were misclassified as diploids. Considering that most seed (>85%) resulting from the induction crosses are diploids, the red root marker is valuable in identifying and discarding large numbers of diploids that are not of any value in DH line production. Comparing the MCC values resulting from the red root marker-based and *R1-nj* marker-based haploid/diploid classifications (overall 0.89 vs. 0.82), it can be further inferred that the red root marker-based classification has a higher correlation with the true ploidy status of the plants than that of the *R1-nj* marker. Taken together, the results from our study clearly demonstrate that the red root marker can be used for recovering high proportion of haploids from the induction crosses that show complete inhibition of *R1-nj* at the early seedling stage. In addition, our findings support the supposition that the red root marker can be similarly used for recovering a maximum number of haploids from induction crosses with partial inhibition of *R1-nj* marker expression. If germplasm with segregation for the anthocyanin color inhibitor gene is employed in induction crosses using inducers with both *R1-nj* and red root markers, a certain proportion of the induced seed showing anthocyanin coloration can be classified into haploids and diploids first based on Navajo phenotype. The other portion of seed with inhibition of the Navajo phenotype can be germinated and the seedlings can be separated into haploids and diploids based on root coloration. Thus, the triple marker haploid inducers could be highly useful for DH line production in tropical maize breeding populations and landraces which show a high frequency of complete/partial inhibition of Navajo marker expression (Chaikam et al., 2015).

The red root marker also paves the way for the efficient identification of false positives in the haploid fraction separated based on *R1-nj* before the application of expensive and laborious chromosomal doubling treatments. Previously, the purple sheath marker was proposed to identify such false positives and some of the inducer lines are equipped with this marker (Röber et al., 2005; Li et al., 2009). Our study indicated that purple sheath occurs naturally at a frequency of ~60% in tropical landraces and ~10% in elite CIMMYT inbreds that are used worldwide. Hence, under many circumstances, this marker could not work for selection in many landraces and populations using inbreds with natural sheath coloration. In addition, this marker cannot identify haploids before chromosomal doubling treatments as it only expresses at later stages of plant establishment. Hence, it can be concluded that the sheath color marker is of little use in DH line development. On the contrary, red root color was not noticed in any tropical inbreds used in this study and occurs rarely (~8.6%) in the landraces. As also demonstrated in this study, the red root marker is expressed in germinating seedlings and is independent of the expression of *R1-nj*. Taken together, it is clear that the red root marker is best suited for the identification of false positives before chromosomal doubling treatments.

In addition, the red root marker can aid in the identification of haploids in the germplasm with natural anthocyanin coloration in the seed. In such germplasm, *R1-nj* expression may be masked and, thus, could not be effective as demonstrated in this study using purple and red corn synthetics. Natural anthocyanin expression in the seed is not present in the inbreds developed at CIMMYT and also in most inbreds developed by public and private maize breeding programs worldwide, which primarily focus on yellow and white maize. Hence, the masking

of *R1-nj* is not a problem in inbreds and populations derived from them. On the contrary, 40.9% of the landraces analyzed in this study showed natural anthocyanin accumulation in the seed. Some of these landrace accessions have significant nutritional and economic value. For example, purple maize landraces are used in some parts of Central and South America for making purple tortillas, drinks such as "*chicha morada*" and also as a source of colorant (Petroni et al., 2014). In addition, the demand for this type of corn is growing worldwide as the health benefits with anthocyanin-rich foods is becoming widely publicized. Hence, there is an opportunity for improving such landraces through modern breeding approaches. The production of inbred lines through DH technology with *R1-nj* based haploid inducers is not possible in such landraces. As anthocyanin coloration occurs rarely in the roots of landrace accessions (~8.6%), the red root marker does not suffer from the masking effects. In this study, using blue corn and red corn synthetics, we demonstrated that the red root marker can effectively identify haploids from such germplasm with reasonably high accuracy as indicated by the MCC value of 0.82. However, some diploids can be misclassified in the haploid fraction as indicated by FDR (overall ~27%). Very few haploids are falsely identified in the diploid fraction using the red root marker as indicated by the FNR (overall ~3.4%).

In summary, the red root marker perfectly complements the *R1-nj* marker in haploid identification in germplasm with complete or partial inhibition of *R1-nj* and in germplasm with natural anthocyanin accumulation in the seed. It also helps to eliminate false positives before chromosomal doubling treatments. Hence, the triple marker inducers equipped with both *R1-nj* and red root marker are valuable to identify haploids in the majority of the germplasm with reasonable accuracy and to increase the overall efficiency of the DH production process. However, one limitation of using red root marker is that it requires the germination of thousands of seeds resulting from haploid induction crosses, which is both labor- and resource- intensive as compared to *R1-nj* or high kernel oil based haploid/diploid classification. To address this problem, we recommend integrating the high oil trait into triple marker inducers, which may help in adopting a pyramiding classification scheme to separate haploids from diploids at different stages of plant development. Using such inducers, the first separation of haploids and diploids can be achieved by using the high oil trait. At the second stage, any diploid seed falsely identified in the haploid fraction can be eliminated using the *R1-nj* marker (if expressed). At the third stage of selection, the red root marker can be employed at the seedling stage. Collectively, these three levels of selection could substantially reduce the cost of labor involved in haploid identification and the rate of false positives in the field. If any false positives escape these three levels of selection, they can be eliminated by using the purple sheath or stem marker or by looking at the plant stature. Until triple marker inducers with high oil trait are available, it would be pragmatic to use a classification scheme based on the *R1-nj* marker and/or high oil content in induction crosses with inducers such as UH600 or UH601 (Melchinger et al., 2013), possessing both of these markers and limit the use of red root marker to those cases where these markers cannot be employed. In the latter case, the germination process needs to be further optimized to reduce the labor and costs associated with this process. In addition, it is also necessary to integrate the red root marker in better-adapted tropical haploid inducers to achieve optimum agronomic performance in the induction nurseries in diverse tropical environments.

Supplemental Materials Available

Supplemental material is available with the online version of this article.

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