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The *RUBY* reporter enables efficient haploid identification in maize and tomato

Dong Wang[1,†], Yu Zhong[1,†], Bin Feng[1], Xiaolong Qi[1], Tongzheng Yan[1], Jinchu Liu[1], Shuwei Guo[1], Yuwen Wang[1], Zongkai Liu[1], Dehe Cheng[1], Yuling Zhang[1], Yunlu Shi[2], Shuaisong Zhang[2], RuXue Pan[2], Chenxu Liu[1,3,*] and Shaojiang Chen[1,3,*]

[1]*National Maize Improvement Center of China, College of Agronomy and Biotechnology, China Agricultural University, Beijing, China*
[2]*Center for Crop Functional Genomics and Molecular Breeding, China Agricultural University, Beijing, China*
[3]*Hainan Yazhou Bay Seed Laboratory, Sanya, China*

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**Correspondence* (Tel +8613811736989 (S.C.); Tel +8618201035243 (C.L);
email chen368@126.com (S.C.);
liucx@cau.edu.cn (C.L.))
†These authors contributed equally to this work.

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Summary

In vivo haploid induction has been extended from maize to monocotyledonous plants like rice, wheat, millet and dicotyledonous plants such as tomato, rapeseed, tobacco and cabbage. Accurate identification of haploids is a crucial step of doubled haploid technology, where a useful identification marker is very pivotal. *R1-nj* is an extensively used visual marker for haploid identification in maize. RFP and eGFP have been shown to be feasible in identifying haploid. However, these methods are either limited to specific species, or require specific equipment. It still lacks an efficient visual marker that is practical across different crop species. In this study, we introduced the *RUBY* reporter, a betalain biosynthesis system, into maize and tomato haploid inducers as a new marker for haploid identification. Results showed that expression of *RUBY* could result in deep betalain pigmentation in maize embryos as early as 10 days after pollination, and enabled 100% accuracy of immature haploid embryo identification. Further investigation in tomato revealed that the new marker led to deep red pigmentation in radicles and haploids can be identified easily and accurately. The results demonstrated that the *RUBY* reporter is a background-independent and efficient marker for haploid identification and would be promising in doubled haploid breeding across different crop species.

Introduction

Doubled haploid (DH) technology has been developed and substantially aided commercial maize (*Zea mays* L.) breeding in the last decade. Induction, identification and chromosome doubling of haploids are crucial steps in DH technology (Ren *et al.*, 2017). Through these processes, homozygous DH lines can be generated in only two generations, as opposed to eight generations via successive self-pollination in conventional breeding (Jacquier *et al.*, 2020). Recently, genes such as *ZmPLA1/MATL/NLD* (Gilles *et al.*, 2017; Kelliher *et al.*, 2017; Liu *et al.*, 2017), *ZmDMP* (Zhong *et al.*, 2019), *ZmPLD3* (Li *et al.*, 2021) and *ZmPOD65* (Jiang *et al.*, 2022) that contribute to *in vivo* haploid induction (HI) have been cloned in maize. These works paved the way for constructing a general HI system in different crop species. By knocking out of orthologues of *ZmPLA1/MATL/NLD* or *ZmDMP*, *in vivo* HI has been proven feasible in plant species like rice (*Oryza sativa* L.) (Yao *et al.*, 2018), wheat (*Triticum aestivum*) (Liu *et al.*, 2020) and foxtail millet (*Setaria italica*) (Cheng *et al.*, 2021), and dicot crops, such as tomato (*Solanum lycopersicon*) (Zhong *et al.*, 2022a), tobacco (*Nicotiana tabacum*) (Zhang *et al.*, 2022a; Zhong *et al.*, 2022b), rapeseed (*Brassica napus*) (Li *et al.*, 2022; Zhong *et al.*, 2022b), alfalfa (*Medicago truncatula*) (Wang *et al.*, 2022), cabbage (*Brassica oleracea*) (Zhao *et al.*, 2022) and potato (*Solanum tuberosum* L.) (Zhang *et al.*, 2022b). These works opened a new era for establishing a general DH technology that can be applied to more crops.

About 90% of the progeny generated by maize haploid inducer were diploids. Accurate haploid identification (HID) is essential before chromosome doubling operation (Ren *et al.*, 2017). *R1-navajo* (*R1-nj*) is one of the most extensively used markers for maize HID because of its clear pigmentation in both scutellum and aleurone (Chaikam *et al.*, 2019; Lashermes and Beckert, 1988; Nanda and Chase, 1966). Among the progeny pollinated by a haploid inducer carrying the *R1-nj* marker, kernels with purple aleurone but without purple pigment in the embryos were regarded as haploids (Nanda and Chase, 1966). However, the application of *R1-nj* is limited, because many tropical and temperate germplasms carry pigmentation inhibitors like *C1-I* and *C2-idf*, which inhibit pigmentation in scutellum of diploid kernels, thus make it difficult to identify haploids (Chaikam *et al.*, 2015; Della Vedova *et al.*, 2005). The oil content has been proposed and proved to be an efficient marker for maize HID at mature kernel stage (Chen and Song, 2003; Melchinger *et al.*, 2013). However, measuring kernel oil content was not appropriate for HID at immature embryo stage or for other crops. Recent studies generated transgenic plants with eGFP, DsRED or TagRFP, which can successfully mark the expected tissues and realize HID in maize (Dong *et al.*, 2018; Yu and Birchler, 2016), *Arabidopsis* (Zhong *et al.*, 2020), tomato (Zhong *et al.*, 2022a), rapeseed (Li *et al.*, 2022; Zhong *et al.*, 2022b) and potato (Zhang

et al., 2022b). Nevertheless, a particular light source and filters were required, and the operation was not as convenient as using visual markers. Over-expression of *ZmC1* and *ZmR2* could realize visible anthocyanin accumulation in both immature embryos and mature kernels of maize (Chen *et al*., 2022a). Still, it is unknown whether the *ZmC1* and *ZmR2* can be used for HID in tiny-seed crops like tomato. Therefore, it is urgent to find a visual marker system for establishing efficient HID in DH breeding across different crop species.

Betalains are tyrosine-derived pigments that constitute one of the major pigment varieties in plants (Clement and Mabry, 1996). It was demonstrated that the biosynthesis of betalain only requires three genes, *CYP76AD1*, *BvDODA1* and *cDOPA5GT* (Polturak *et al*., 2016). In a recent study, the open reading frame containing the three genes was named as *RUBY* and used as a reporter to monitor gene expression and plant transformation (He *et al*., 2020). The application of betalain in generating a plant-made pink cotton fibre indicated that the *RUBY* worked well in plant tissues of interest (Ge *et al*., 2023; Li *et al*., 2023). Given the vivid colour and easily-synthesis of betalain, we hypothesized that the *RUBY* reporter can be used as a visual marker in HID across different crop species.

In this study, we demonstrated that the *RUBY* reporter enables accurate HID in maize and tomato through betalain accumulation in embryos and radicles. The *RUBY* reporter would be a general and promising marker system in DH breeding of different crop species.

Results

Development and characterization of maize RUBY haploid inducer

To achieve betalain accumulation in different growth stages, we chose the *ZmUBI* promoter to drive *RUBY* expression in maize (Figure 1a). After transformation, six positive transgenic events were obtained. Transgenic events #1, #2 and #3 showed normal colour in leaves, stems and kernels (Table S1), these events, thus were abandoned. Transgenic events #4 and #5 exhibited green and red striped leaves, green stem and deep red seeds, #6 had strong red pigmentation in stems, leaves and seeds (Table S1). The transgenic event #5 was crossed with the haploid inducer line, CHOI4. Crossed seeds carrying *RUBY* marker were planted and then self-pollinated to obtain F_2. In F_2, all the plants were genotyped and self-pollinated. Three individuals with heterozygous *zmpla1*, *zmdmp* and *RUBY* were selected to obtain F_3. By checking kernels on each self-pollinated ear of the F_3 generation, we identified an individual, with homozygous *zmpla1*, *zmdmp* and *RUBY* alleles, as the RUBY haploid inducer (also written as RUBY inducer) (Figure 1b). It exhibited deep red-violet colour in stem, leaves, tassels (Figure 1b), 15 days after pollination (DAP) embryos (Figure 1c), germinated roots and shoots (Figure 1d,e), ear (Figure 1f) and seedling (Figure 1g). And the embryo pigmentation in mature kernel of RUBY inducer is dramatically stronger than that of CHOI4 (Figure 1h,i).

Haploid identification with *RUBY* reporter in maize

To evaluate the performance of the *RUBY* marker in outcrossing, ZD958 was used as a female parent to cross with the RUBY inducer. The mature crossed kernels were red (Figure 1h). Vertical sections of the kernels showed that betalain pigments mainly accumulated in embryo and aleurone, and few pigments were observed in endosperm (Figure 1h). Among the crossed kernels, we also identified kernels with red aleurone and colourless embryos, these kernels were deemed as haploids (Figure 1h). Compared with the diploid kernel derived from ZD958 × CHOI4,

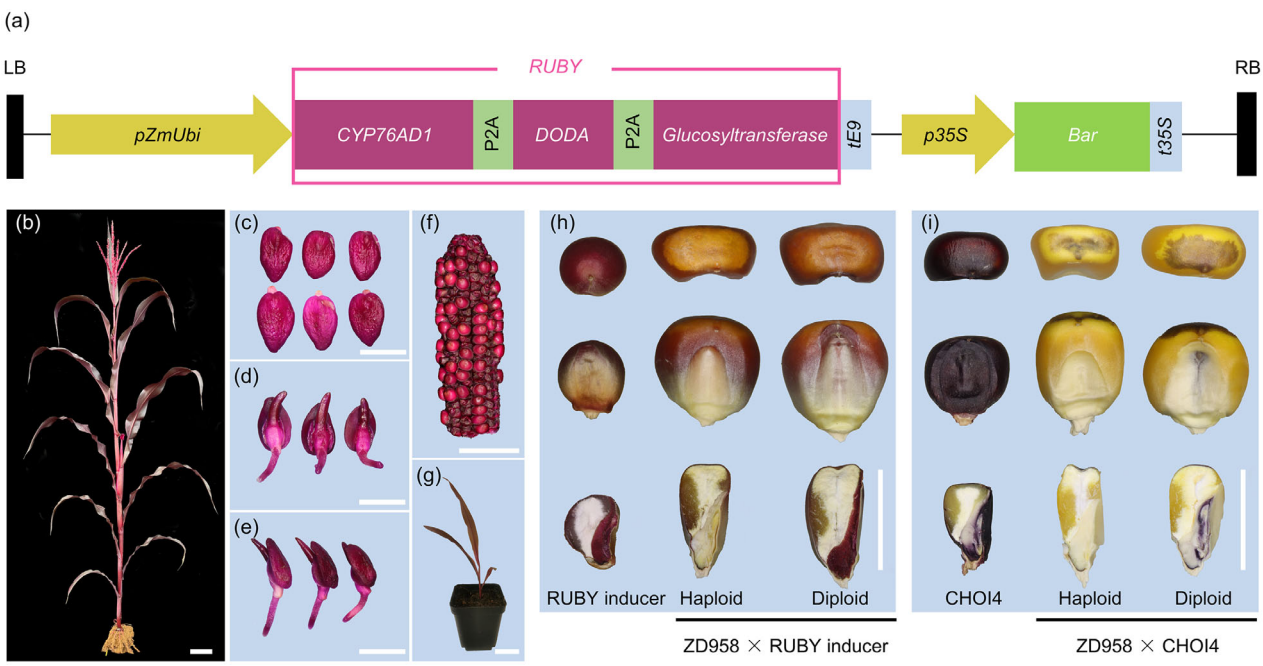

Figure 1 Development and characterization of maize RUBY haploid inducer. (a) A schematic of *RUBY* expression construct in maize. Three betalain biosynthetic genes *CYP76AD1*, *DODA* and *Glucosyltransferase* were linked with 'self-cleaving' 2A peptides and driven by *ZmUbi* promoter. *pZmUbi*, *ZmUbi* promoter; P2A, 'self-cleaving' 2A peptides; *tE9*, E9 terminator; *p35S*, *CaMV 35S* promoter; *t35S*, *CaMV 35S* terminator. (b–g) Pigmentation of plant (b), immature embryos (c), germinated embryos (d, e), ear (f) and seedling (g) of RUBY haploid inducer. (h, i) The mature seeds of haploid and diploid from ZD958 × RUBY haploid inducer (h) and CHOI4 (i), respectively. Scale bars, b, 10 cm; c-e, 0.5 cm; f and g, 3 cm; h and i, 1 cm.

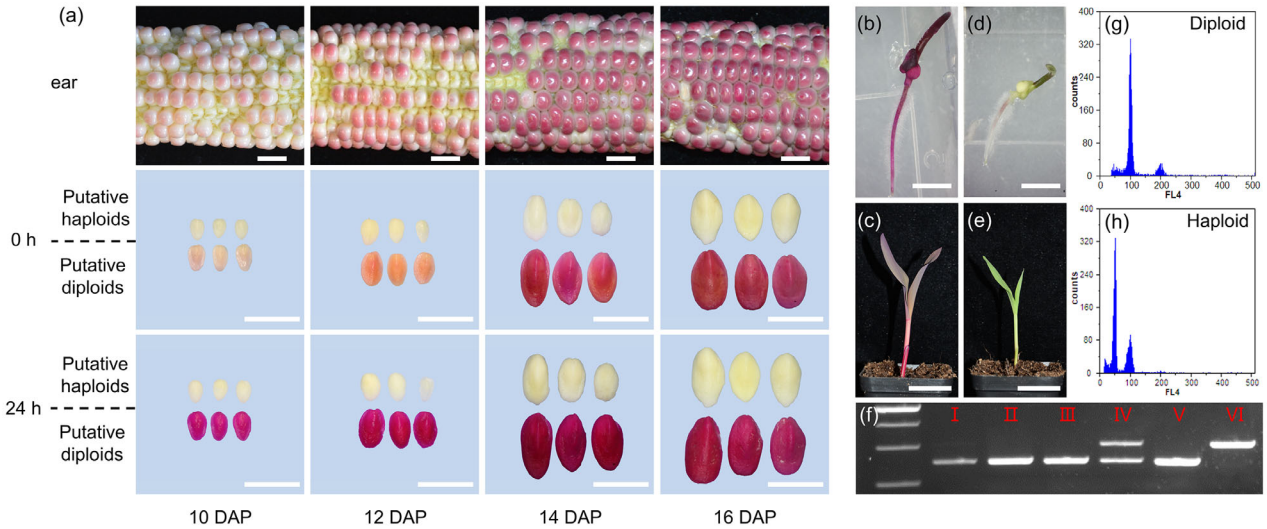

Figure 2 Haploid identification in immature embryos with maize RUBY haploid inducer. (a) Ears and embryos from ZD958 × RUBY haploid inducer at 10, 12, 14 and 16 days after pollination (DAP) and under *in vitro* culture for 0 h and 24 h. (b–e) Characteristics of a diploid seedling from the red embryo (b, c) and a haploid seedling from the white embryo (d, e). (f) Genotyping of putative haploids with polymorphic molecular marker Chr4-97.5. Haploids presented one band from ZD958, while diploid showed two bands from both parents. I–III, haploids, IV, diploid, V, ZD958, VI, RUBY haploid inducer. (g, h) Flow cytometry results of diploid (g) and haploid (h). Scale bars, a (for embryos), b, d, 0.5 cm; a (for ears), 1 cm; c and e, 3 cm.

the pigmentation of embryos from ZD958 × RUBY inducer was much stronger (Figure 1h,i).

We were curious about when the pigment accumulation in embryos was enough to identify haploids. To this end, immature embryos from ZD958 × RUBY inducer were isolated and checked at different DAPs. The colour of diploid embryos changed from light pink to orange from 10 to 12 DAP, and continued intensifying to red at 14 DAP (Figure 2a). There was little change in embryo colour from 14 to 16 DAP (Figure 2a). According to the pigmentation, immature embryos from the four stages were classified clearly into two categories, putative diploids (Figure 2a–c) and putative haploids (Figure 2a,d,e). Both roots and seedlings from the putative diploid embryos were red (Figure 2b,c). While in the control panel, the embryos from ZD958 × CHOI4 were colourless from 10 to 16 DAP (Figure S1). Genotyping results with polymorphic markers indicated that all 66 putative haploids showed maternal genotype, and 768 putative diploids were heterozygous (Figure 2f). Among the 66 putative haploids with narrow and erect leaves, 20 individuals were chosen randomly for ploidy analysis with flow cytometry. Results showed that all of them were true haploids (Figure 2g,h). Combining the genotyping, phenotyping and flow cytometry results, the haploid identification accuracy was 100% and false negative rate was 0 (Table 1). Compared to the regular haploid inducer, expression of *RUBY* could enable accurate haploid identification as early as 10 DAP.

Previous studies revealed that embryo pigmentation was further enhanced after *in vitro* culture, especially under strong light (Chen *et al.*, 2022b). We were curious about whether betalain pigment accumulation could also be facilitated by *in vitro* culture. To this end, we compared the pigmentation intensity before and after *in vitro* culture. After *in vitro* culture for 24 h, the pigmentation of embryos harvested at 10, 12 and 14 DAP was significantly intensified while little difference was observed for those harvested at 16 DAP (Figure 2a). On the contrast, the 10–16 DAP embryos derived from ZD958 × CHOI4 did not show anthocyanin accumulation after 24 h culture (Figure S1). To further confirm the impact of light exposure on betalain accumulation in immature embryos, we compared the pigmentation of 12-DAP embryos cultured in light and dark environments. Results showed that betalain pigments could be enhanced similarly in both environments after 12 h *in vitro* culture (Figure S2). More importantly, the pigment mainly accumulated in the first 12 h of *in vitro* culture (Figure S2), demonstrating that the betalain could accumulate rapidly during *in vitro* culture and light exposure was not a necessary factor.

Table 1 The haploid identification performance of *RUBY* in maize immature embryos

DAP	Putative diploids	True diploids	Putative haploids	True haploids	Accuracy (%)	FNR (%)
10	180	180	13	13	100	0
12	203	203	15	15	100	0
14	173	173	18	18	100	0
16	212	212	20	20	100	0
Sum	768	768	66	66	100	0

FNR, false negative rate; Accuracy (%) = number of true haploid/number of putative haploid ×100%; FNR (%) = number of haploid from putative diploids/number of putative diploid × 100%.

Many tropical and temperate germplasms carry *R1-nj* inhibitors, like *C1-I*, which hinders anthocyanin accumulation and results in weak-colour or colourless kernels (Chaikam *et al.*, 2015). To test the performance and stability of *RUBY* in these *C1-I* germplasms, we crossed the homozygous RUBY haploid inducer, CHOI4 and MAGIC1, respectively, to a *C1-I* inbred line, Zong31. At 10 DAP, putative diploid embryos from Zong31 × RUBY inducer showed light red at 0 h, but showed deep red after *in vitro* culture for 24 h (Figure 3a). While the 10-DAP embryos from Zong31 × CHOI4 and Zong31 × MAGIC1 did not exhibit visible anthocyanin, even after *in vitro* culture for 24 h (Figure 3a). At 16 DAP, diploid

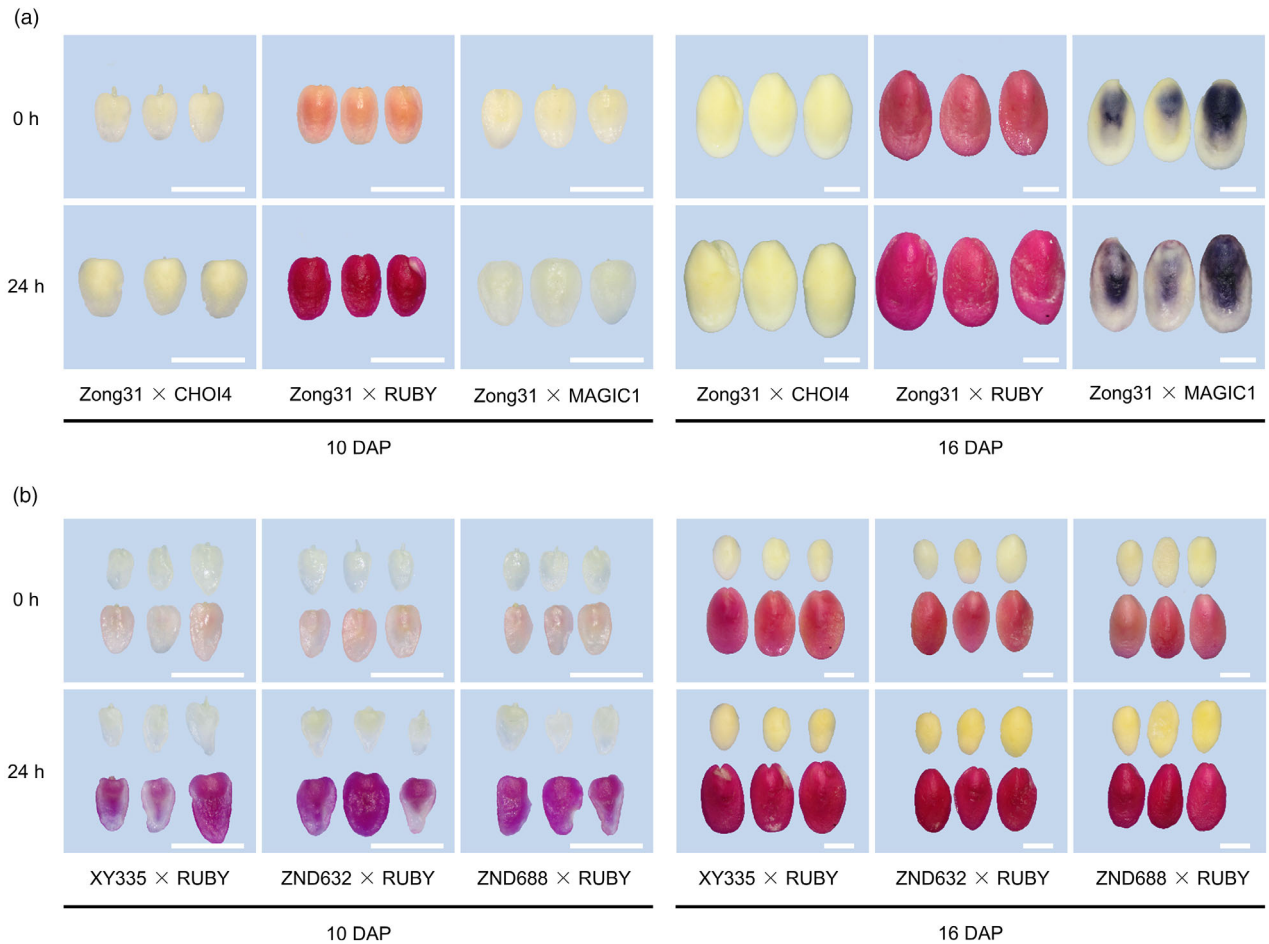

Figure 3 *RUBY* functions in multiple germplasms of maize. (a) Pigmentation performance of diploid immature embryos from Zong31 × CHOI4, Zong31 × RUBY haploid inducer and Zong31 × MAGIC1, at 10 DAP and 16 DAP, after *in vitro* culture for 0 h and 24 h, respectively. (b) Immature embryos from XY335, ZND632 and ZND688 × RUBY haploid inducer at 10 DAP and 16 DAP and under *in vitro* culture for 0 h and 24 h, respectively. DAP, days after pollination. Scale bars, 0.25 cm.

embryos from Zong31 × RUBY inducer showed deep red-violet and were more evident than that at 10 DAP (Figure 3a). The 16-DAP embryos from Zong31 × CHOI4 remained colourless (Figure 3a). 16-DAP embryos from Zong31 × MAGIC1 showed good pigmentation performance, but anthocyanin was not evenly distributed in embryos (Figure 3a). The diploid and haploid embryos from hybrids XY335, ZND632 and ZND688 that pollinated by RUBY inducer exhibited distinct coloration at 10 and 16 DAP (Figure 3b). These results indicated that betalain could conquer the obstacle of pigmentation inhibitor and function well in different germplasms.

Development and characterization of tomato RUBY haploid inducer

In tomato, *RUBY* was expressed under the control of *CaMV 35S* promoter (Figure 4a). After transformation to a tomato haploid inducer with homozygous alleles of *sldmp*, 21 transgenic events were obtained. According to the colour of leaf, stem, seed, radicle and seedling, the 21 events were divided into 10 different types (Table S2). Considering the hairy seed coat of tomato seeds, pigmentation in radicles may make it easier for examination and more effective in HID. Therefore, we examined all transgenic events and found four of them, #12, #13, #20 and #21, with clear red radicles. Among the four transgenic events, #20 and #21 showed strong red pigmentation in all tissues and retarded growth, and were abandoned (Table S2). Transgenic events #12 and #13 showed normal in plant colour and growth, and were self-pollinated to obtain homozygous RUBY inducer (Table S2). In T_1, a homozygous RUBY inducer (#13-5) was screened from event #13 and self-pollinated. Among the progeny derived from #13-5, one individual (#13-5-6) was chosen as a candidate RUBY inducer (Figure 4b). The tomato RUBY inducer showed deep red in seeds, germinating radicles, hypocotyl and cotyledons (Figure 4g–j). The pigmentation of radicles was intensifying along growth (Figure 4h,i). With the growth of plant, only the bottom leaves kept light red, the leaves in middle and top of the plant were green (Figure 4b,c). The colour of fruits from RUBY inducer was similar to that of wild-type plants (Figure 4e,f), except for the flowers, which showed light red in the base part of petals (Figure 4d). The seeds, germinating roots, cotyledons and hypocotyl of the control plant, Alisa Craig (AC), were normal in colour (Figure 4k–n).

Haploid identification with *RUBY* reporter in tomato

To evaluate the HID performance of *RUBY*, a hybrid F_1 (Alisa Craig × MicroTom), two inbred lines AC and M82, were used as female

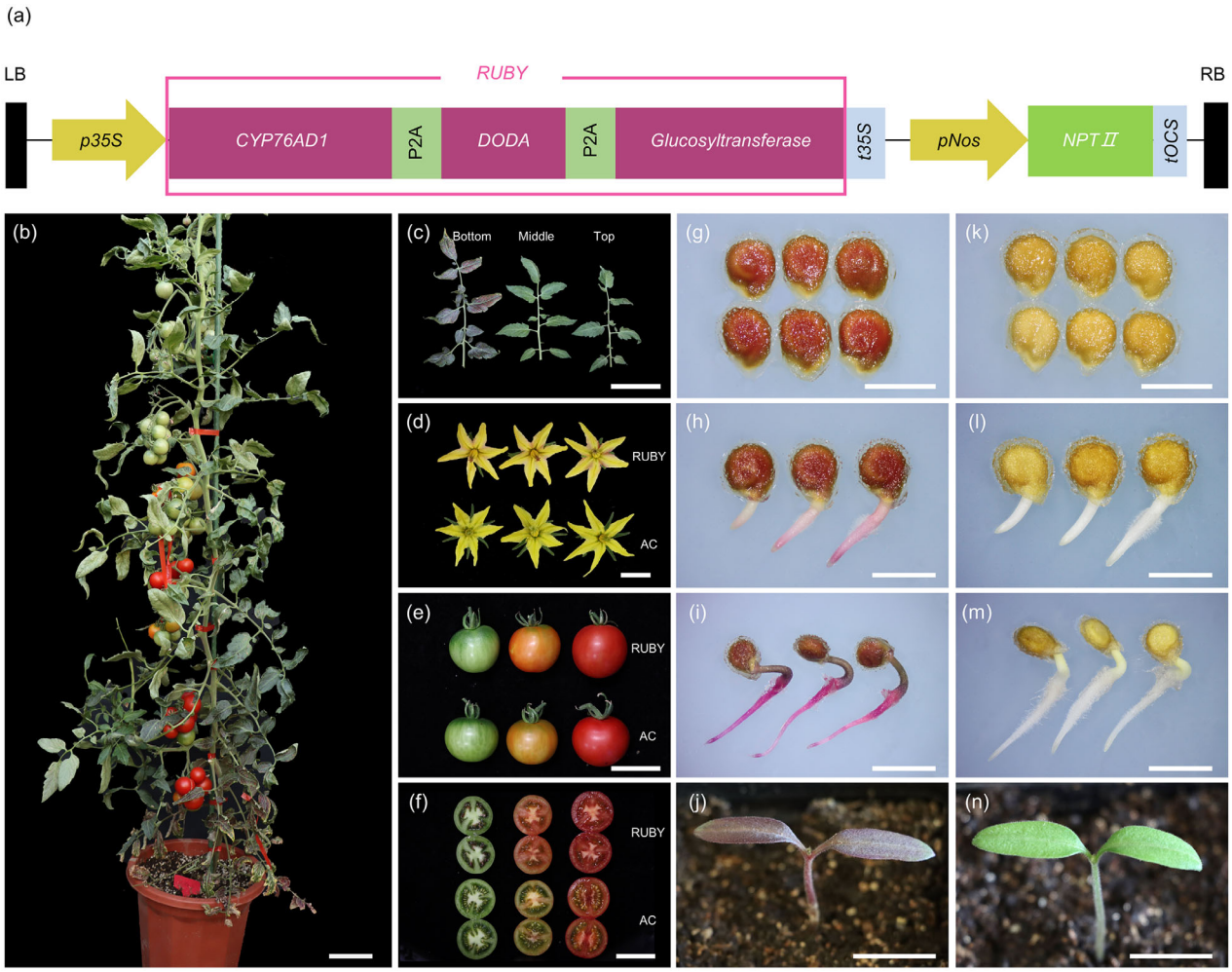

Figure 4 Development and characterization of tomato RUBY haploid inducer. (a) A schematic of *RUBY* expression vector in tomato. *p35S*, *CaMV 35S* promoter; P2A, 'self-cleaving' 2A peptides; *t35S*, *CaMV 35S* terminator; *pNOS*, *Nopaline synthase* promoter; *tOCS*, *Octopine synthase* terminator. (b) A candidate tomato RUBY haploid inducer plant. (c) The bottom, middle and top leaves of RUBY haploid inducer. (d) The flowers of RUBY haploid inducer (up) and Alisa Craig (down). (e, f) The fruits of RUBY inducer and Alisa Craig at the green (left), pink (middle) and red (right) stages of ripening. (g–i) *RUBY* functions in tomato seeds and radicles. (j) The seedling of RUBY haploid inducer with red cotyledons and hypocotyl. (k–m) The seeds and radicles of Alisa Craig. (n) The seedling of Alisa Craig with green cotyledons and white hypocotyl. Scale bars, b and c, 10 cm; d, g–n, 1 cm; e and f, 4 cm.

parents to cross with the tomato RUBY inducer. For F_1, AC and M82, we harvested 797, 300 and 561 seeds, respectively (Table 2). All seeds were red compared to wild-type, and haploid could not be identified according to seed colour (Figure 5a).

After germination, pigmentation was observed in radicles (Figure 5a). With the growth of roots, red radicles showed stronger pigmentation and were more distinct from white ones. Seeds with red roots were considered as putative diploids (Figure 5b) and these with white roots were deemed to be putative haploids (Figure 5c). Among the crossed progenies of F_1, AC and M82, we identified 11, 3 and 10 putative haploids, respectively (Table 2). Subsequent analysis with molecular markers confirmed that all putative haploids were maternal origin and all putative diploids were heterozygous (Figure 5d). The ploidy level of 24 haploids and some diploids was further verified by flow cytometry analysis, results showed that all of them were true haploids and diploids, respectively (Figure 5e,f, Table 2). Taken together, introduction of *RUBY* reporter to haploid inducers could achieve accurate HID in tomato.

Table 2 The haploid identification performance of *RUBY* in tomato

Female parent	Putative diploids	True diploids	Putative haploids	True haploids	Accuracy (%)	FNR (%)
F_1	786	786	11	11	100	0
AC	297	297	3	3	100	0
M82	551	551	10	10	100	0
Sum	1634	1634	24	24	100	0

F_1, the hybrid (Alisa Craig × MicroTom); AC, Alisa Craig.

Discussion

Advantages of *RUBY* in HID

HID is a crucial process in DH breeding (Ren *et al.*, 2017). The efficiency and accuracy of HID were mainly decided by marker from haploid inducers (Prasanna, 2012). *R1-nj* is still an extensively used HID marker in maize (Chaikam *et al.*, 2019).

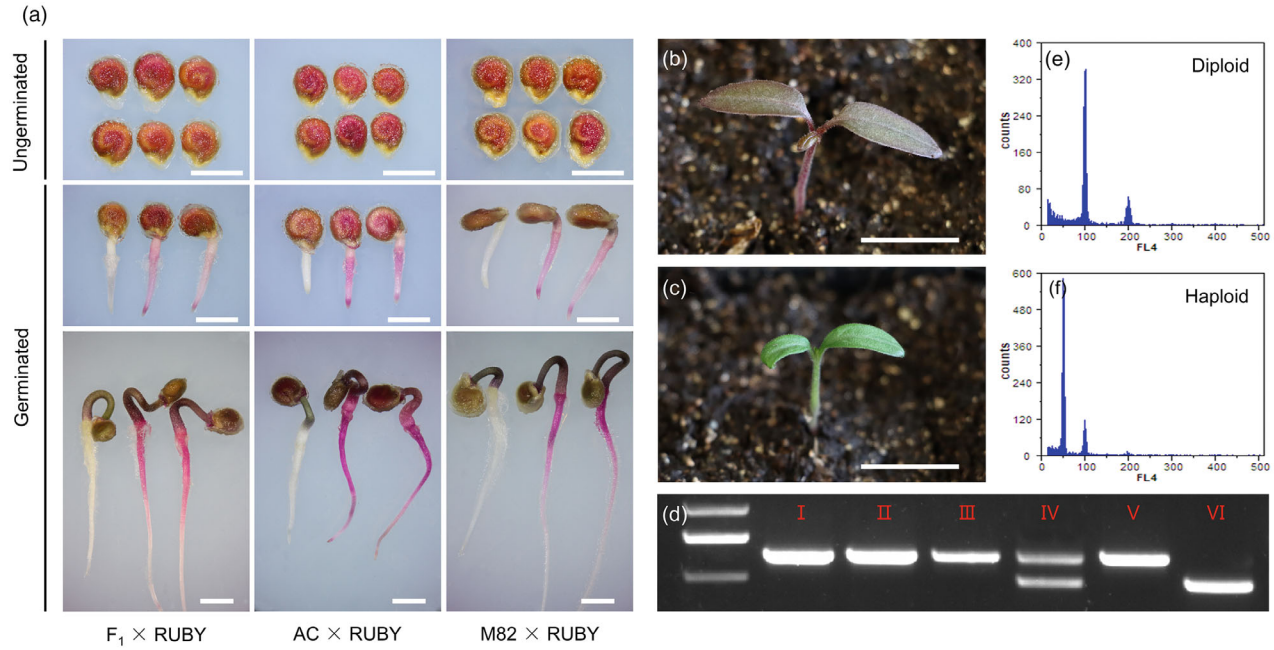

Figure 5 Haploid identification with tomato RUBY haploid inducer. (a) Haploid identification through recognizing the colour of germinated radicles. Each column represents the female parent F_1 (Alisa Craig × MicroTom), Alisa Craig and M82, respectively. Different stages of germination are displayed in rows. (b, c) The putative diploid (b) and haploid (c) from F_1 × RUBY haploid inducer. (d) Genotyping of putative haploids and diploids through polymorphic molecular marker Solyc05g007920. Haploids presented one band from F_1 while diploid showed two bands from both parents. I-III, haploids; IV, diploid; V, F_1 (Alisa Craig × MicroTom); VI, RUBY haploid inducer. (e, f) Flow cytometry results of diploid (e) and haploid (f). Scale bars, 1 cm.

Recently, the development of immature embryo-based chromosome doubling in maize has further improved the efficiency of chromosome doubling, but also requires the HID at immature embryo stage (Chen *et al.*, 2022b). The application of *R1-nj* marker in immature embryo HID required isolation of all the embryos and followed by light exposure for 24–48 h. The work would be dramatically decreased if haploid embryos could be immediately distinguished during embryo isolation. Therefore, efficient HID at immature embryo stage would be helpful to further improve DH breeding efficiency. Previous studies revealed that over-expression of *ZmC1* and *ZmR2* resulted in anthocyanin accumulation in maize immature embryos at 12 DAP (Chen *et al.*, 2022a). Nevertheless, anthocyanin synthesis requires a series of genes, and it is unknown whether *ZmC1* and *ZmR2* can be used in tomato, rapeseed, and other crop species.

In this study, we introduced the *RUBY* into maize and found that haploid embryos could be identified as early as 10 DAP, thus would be very useful in immature embryo *in vitro* culture-based chromosome doubling and DH production. Meanwhile, we have also demonstrated that *RUBY* could result in tomato tissue pigmentation, and successfully screened a transgenic event with intense pigmentation in germinating radicles. Further analysis revealed that *RUBY* reporter could achieve accurate HID in both maize and tomato, without false negative cases, thus would be promising in DH breeding of different crop species.

More importantly, *C1-I* exists in many maize germplasms, and would inhibit the biosynthesis of anthocyanin (Chaikam *et al.*, 2015; Paz-Ares *et al.*, 1990). The application of *RUBY* is theoretically not affected by *C1-I*, because only three genes are involved in betalain synthesis from the substrate, tyrosine (Polturak *et al.*, 2016). Generally, tyrosine is widely distributed in different cell types (He *et al.*, 2020). Therefore, the inhibitor *C1-I* is unable to prevent the synthesis of betalain.

In comparison with the TagRFP or double-fluorescence markers previously reported that was equipment and experience dependent (Dong *et al.*, 2018; Zhong *et al.*, 2020, 2022b), using *RUBY* to identify haploids was visible, and thus was more convenient in operation. In addition, given the strong and stable betalain pigments of *RUBY* marker in both maize and tomato, the application of *RUBY* marker in HID would lay a solid foundation for automatic and high-throughput HID with machine vision technology.

Optimization of *RUBY* HID system

During the development of maize and tomato transgenic lines, we chose Zm*Ubi* and *CaMV 35S* promoters to drive the *RUBY*, respectively. The transgenic events of maize and tomato showed varied pigmentation patterns (Tables S1, S2). We observed that excessive accumulation of betalain in leaves, flowers and fruits resulted in a series of detrimental phenotypes in tomato, including retarded growth, reduced pollen amount and low fruits setting rate (Figure S3). But in our observation, the expression of betalain in maize leaves had a limited effect on its growth. Compared with *R1-nj* which is expressed only in part of the aleurone, the accumulation of betalain in aleurone made it difficult to observe the colour of embryos at mature seed stage, and disadvantageous for haploid kernel selection in maize (Figure 1h). To further improve the performance of *RUBY* in HID, screening of promoters with proper expression level and specific expression pattern is essential. In a previous study, promoter optimization could reduce the negative effect of betalain accumulation in tomato plant and control the expression

section (Polturak *et al*., 2017). Here, we suggest that, in crop species like maize and wheat, the embryo-specific homologous promoters like promoter of *pOLEO1* may be ideal (Zhong *et al*., 2020). In these tiny-seed species like tomato, rapeseed, tobacco, and these species with glumes like rice, radicle-specific expressed promoters like *pOsSli2* (Chen *et al*., 2015; Ma *et al*., 2007) may function well.

In conclusion, our results demonstrated for the first time that the *RUBY* reporter could be used for efficient and accurate HID in maize and tomato, and would also be promising in DH breeding in more crop species.

Experimental procedures

Plant materials and growth conditions

In maize, the inbred line ND101 was used in *Agrobacterium*-mediated transformation. Two haploid inducer lines, CHOI4 and MAGIC1, developed by CAU, were used in this study (Chen *et al*., 2022a; Liu *et al*., 2022). CHOI4 is a regular inducer with *R1-nj* marker, which grants visible anthocyanin accumulation in the aleurone layer and the scutellum of kernel. MAGIC1 is a transgenic inducer, which has an anthocyanin marker generated from co-expression of two transcription factor genes (*ZmC1* and *ZmR2*) driven by the embryo–aleurone-specific bidirectional promoter *pZmBD1* (Liu *et al*., 2018). To evaluate the performance of *RUBY* in HID, four commercial hybrids, ZhengDan958 (ZD958), ZhongNongda688 (ZND688), ZhongNongda632 (ZND632) and XianYu335 (XY335), and one inbred line with *C1-I*, Zong31 were planted as female testers. Both CHOI4 and MAGIC1 were used as the control to compare the performance of *RUBY* in HID. In tomato, we chose a tomato haploid inducer, *sldmp* mutant (157 bp deletion at *SlDMP* and Cas9-free) in Alisa Craig background, to do transformation. The hybrid, F_1 (Alisa Craig × MicroTom) and two inbred lines, Alisa Craig (AC) and M82 were used as testers. AC was used as male parent for control. Transgenic plant nursing was carried out in growth chamber under photoperiod and temperature control of 16 h under light at 26 °C, and 8 h in the dark at 23 °C in the first generation, and then were planted in open field in the following generations.

Plasmids construction

To generate the *RUBY* reporter construct, the open reading frame of *CYP76AD1*, *DODA* and *Glucosyltransferase* were amplified from *pDR5:RUBY* (He *et al*., 2020) with RUBY-1, RUBY-3 and RUBY-5 primer pairs, respectively. They were cloned into vector pICH41308, together with two P2A sequences formed by annealing oligos of RUBY-2 and RUBY-4. In the *DR5:RUBY* construct, F2A peptides, with the sequence of QLLNFDLLKLAGDVESNPGP, were used to link the three betalain biosynthetic genes. We replaced F2A peptides with P2A peptides, which has the sequence of GSGATNFSLLKQAGDVEENPGP, to achieve higher cutting efficiency. The *RUBY* driven by *ZmUBI* promoter and *CaMV 35S* promoter-expressed *Bar* were fused into the vector pISCL4723 by Golden Gate cloning method to yield *ZmUBI:RUBY* construct (Engler *et al*., 2014). To generate *35S:RUBY* construct for dicotyledon species, *CaMV 35S* promoter-expressed *RUBY* and *Nopaline synthase* (*Nos*) promoter-expressed *NPTII* were introduced simultaneously in one step into pISCL4723 through the Golden Gate cloning.

The development of RUBY haploid inducers

The *ZmUBI:RUBY* construct was transformed to ND101 by *Agrobacterium*-mediated transformation. Positive transgenic events were verified by pigmentation observation and molecular marker RUBY-seq9. Transgenic events were crossed to CHOI4, to introduce *RUBY* marker into haploid inducer line. The kernels with *RUBY* marker were selected for planting and self-pollination in the next generation, to get individual with homozygous *RUBY*. Marker-assisted selection (MAS) of *zmpla1* and *zmdmp* alleles was used during the development of RUBY inducer with primers zmpla1, ZmPLA1 and zmdmp (Table S3). The method of MAS was elucidated by Chen *et al*. (2022a).

In tomato, *Agrobacterium*-mediated transformation of *sldmp* mutant was carried out with *GV3101* strain (Brooks *et al*., 2014). Positive events were verified by molecular marker RUBY-seq2 (Table S1).

Immature haploid embryo identification in maize

The testers were emasculated at the tasselling stage. Ears were bagged 5–7 days before silking to avoid contamination. Each RUBY haploid inducer was used to cross with at least three tester ears to obtain enough progenies. To detect when haploid embryos can be identified by *RUBY* after pollination, we isolated the immature embryo at 10, 12, 14 and 16 days after pollination (DAP) for ZD958 × RUBY haploid inducer and ZD958 × CHOI4. In order to investigate the influence of light exposure on pigmentation accumulation in immature embryo during *in vitro* culture, we chose 12-DAP embryos from ZD958 × RUBY haploid inducer, and carried out 0, 12 and 24 h (h) *in vitro* culture under light exposure or in a dark room to observe the change of coloration. All of the embryos were cultured on 1/2 MS solid medium (2.22 g MS base (M519, PhytoTech), 30 g sucrose and 8 g agar per litre, pH = 5.8, steam sterilized), with the embryo axis up and the scutellum side in contact with the medium. The culture was under the white light of 10 000 Lux, 26 °C and approximate 40% relative humidity (RH) except for mentioned individually. After 0, 24 and 48 h of culture, embryos were photographed with LEICA S6D. Seedlings were transplanted to soil at about V1 stage.

Tomato seeds germination

Fresh tomato seeds were collected from ripe fruits and then were immersed in 3 M HCl for 30 min, in 10% Na_3PO_4 for 20 min and in 2% NaClO for 15 min, with water flushing before every following treatment, to remove the pulp and pectin and sterilize. Seed germination was carried on a wet filter paper inside a petri dish in a dark growth chamber for 2–3 days at 23 °C. The radicle colour was checked visually and photographed with LEICA S6D. Putative haploid and diploid seeds were classified according to the colour of their roots. All plants were transplanted to soil for subsequent verification.

Haploid verification through molecular markers

DNA was extracted from the leaves of putative haploids and diploids through CTAB method (Murray and Thompson, 1980). In maize, two groups of molecular markers were used to analyse the genetic origin of haploids. Chr4-95.7, a polymorphic marker between the maize RUBY haploid inducer and ZD958, was used for examining the existence of DNA from the RUBY haploid inducer (Table S3). Another group of markers (Chr1-8.0, Chr2-2.48 and Chr3-5.3) was polymorphic between two parents of the hybrid ZD958 (Table S3). Theoretically, a haploid would be homozygous at all these three markers. In tomato, putative haploids were verified with four markers, Solyc05g007920, Indel5684, Indel56760 and Indel162692. Solyc05g007920 was

polymorphic between tomato RUBY haploid inducer and testers, while the others were polymorphic between Alisa Craig and MicroTom and were only used in the progeny of F_1 female tester (Table S3). These samples with two bands from any of the molecular markers were deemed diploids while those with single band for all markers were deemed as putative haploids and would be further verified by flow cytometry.

Flow cytometry

About 200 mg young (5 cm^2) leaf tissues were sampled and chopped with a sharp blade for 60 s in 500 μL Nuclei Extraction buffer (Sysmex Partec, Muenster, Germany). Samples were then filtered through a 50 μm filter. Afterwards, 2000 μL DAPI buffer was added to each sample and stained for 2 min in the dark. Nuclei suspension was analysed by a CyFlow Space Flow Cytometer (Sysmex Partec, Muenster, Germany) and the corresponding FloMax software. Diploid maize or tomato samples were used as controls and the position of their first signal peak was set at ~100 (FL4-A value). The samples with the first signal peak at ~50 (FL4-A value) were identified as haploids.

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Conflict of interest

The authors declare no competing interests.

Author contributions

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

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Pigmentation performance of *R1-nj* marker in crossed immature embryos.

Figure S2 The effect of light exposure on betalain pigment accumulation in immature embryos crossed by RUBY haploid inducer.

Figure S3 The negative effect of excessive betalain accumulation on tomato growth.

Table S1 The colour of leaf, stem and seed of positive transgenic maize events.

Table S2 The colour of leaf, stem, seed, radicle and seedling of positive transgenic tomato events.

Table S3 List of primers used in this study.

