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RESEARCH ARTICLE

Establishment of a transformation system in close relatives of wheat under the assistance of *TaWOX5*

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Abstract

Species closely related to wheat are important genetic resources for agricultural production, functional genomics studies and wheat improvement. In this study, a wheat gene related to regeneration, *TaWOX5*, was applied to establish the *Agrobacterium*-mediated transformation systems of *Triticum monococcum*, hexaploid triticale, and rye (*Secale cereale* L.) using their immature embryos. Transgenic plants were efficiently generated. During the transformation process, the *Agrobacterium* infection efficiency was assessed by histochemical staining for β-glucuronidase (GUS). Finally, the transgenic nature of regenerated plants was verified by polymerase chain reaction (PCR)-based genotyping for the presence of the *GUS* and *bialaphos resistance* (*bar*) genes, histochemical staining for GUS protein, and the QuickStix strip assay for bar protein. The transformation efficiency of *T. monococcum* genotype PI428182 was 94.4%; the efficiencies of four hexaploid triticale genotypes Lin456, ZS3297, ZS1257, and ZS3224 were 52.1, 41.2, 19.4, and 16.0%, respectively; and the transformation efficiency of rye cultivar Lanzhou Heimai was 7.8%. Fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) analyses indicated that the *GUS* transgenes were integrated into the distal or near centromere (proximal) regions of the chromosomes in transgenic *T. monococcum* and hexaploid triticale plants. In the transgenic hexaploid triticale plants, the foreign DNA fragment was randomly integrated into the AABB and RR genomes. Furthermore, the transgene was almost stably inherited in the next generation by Mendel's law. The findings in this study will promote the genetic improvement of the three plant species

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for grain or forage production and the improvement of cereal species including wheat for functional genomics studies.

Keywords: *Triticum monococcum*, hexaploid triticale, rye, *TaWOX5*, *Agrobacterium*, transformation efficiency

1. Introduction

Genetic engineering techniques such as genetic transformation mediated by *Agrobacterium* and gene editing based on clustered regularly interspaced short palindromic repeats associated nuclease 9 (CRISPR/Cas9) have become important approaches for improving agronomic and quality traits and stress resistance in cereal plants (Rodriguez-Leal *et al.* 2017; Yang *et al.* 2017; Zhang *et al.* 2018, 2021; Rao and Wang 2021). In recent years, efficient transformation systems have been established in many cereal plants, including wheat (*Triticum aestivum* L., 2n=6x=42, AABBDD), barely (*Hordeum vulgare* L., 2n=2x=14, HH), rice (*Oryza sativa* L., 2n=2x=24), and maize (*Zea mays* L., 2n=2x=20), which greatly accelerates the modification process of these crops by transgenic and gene editing strategies (Hiei *et al.* 1994; Ishida *et al.* 1996; Kumlehn *et al.* 2006; Wang *et al.* 2022). However, it is still difficult to generate transgenic plants from the main plant species related to wheat such as *Triticum monococcum* L. (2n=2x=14, AA), *Triticum tauschii* (2n=2x=14, DD), *Aegilops speltoides* (2n=2x=14, SS), rye (*Secale cereal* L., 2n=2x=14, RR), and hexaploid triticale (2n=6x=42, AABBRR). The wild relatives of wheat are rich in species and diverse in types, and they harbor excellent characteristics including high protein content and resistances to cold, drought, saline–alkali, and various diseases. Therefore, they are an important component of wheat genetic resources and the main donors of excellent exogenous genes for wheat breeding (Pour-Aboughadareh *et al.* 2021).

Wheat is a heterohexaploid plant containing the A, B, and D genomes. Previous studies have shown that the A and D genomes were derived from *T. monococcum* and *T. tauschii*, respectively, while the origin of the B genome is still controversial, although it may have originated from *Ae. speltoides* (Shewry 2009). Cultivated einkorn wheat (*T. monococcum* L. ssp. *monococcum*, 2n=2x=14, A^mA^m) is closely related to *T. monococcum* (also called as urartu wheat) and easy to cross with wheat (Molnár-Láng *et al.* 2015). During the long evolutionary process, abundant genetic resources and numerous favorable genes, such as the ones controlling disease resistances to leaf rust, stripe rust, and powdery mildew (PM), have accumulated in cultivated *T. monococcum* (Zaharieva and Monneveux 2014). Due to the complicated genome of wheat, many genes exist in its genome with at least three copies, which makes the study of gene functions or functional genomics in wheat difficult. The identification of wheat genes in either one of its three ancestral species would make function analysis more convenient. However, transgenic *T. monococcum* plants were obtained by the particle gun-mediated method at a very low efficiency of only 0.0–0.6% (Miroshnichenko *et al.* 2018). Therefore, it is necessary to establish effective transformation systems for the three ancestors to facilitate their genetic improvement and wheat functional genomics research.

Rye is a gramineous species and a valuable food and forage crop. It is also an important germplasm resource for wheat improvement that is highly resistant to abiotic stresses (saline–alkali, drought and cold), and well adapted to low-fertility soils (Crespo-Herrera *et al.* 2017; Li *et al.* 2021). Through hybridization and chromosome translocation, many available genes have been introduced into wheat from rye (Li *et al.* 2022). Hexaploid triticale is a synthetic plant species, which was produced by crossing *Triticum durum* (2n=4x=28, AABB) and rye, followed by chromosome doubling with colchicine treatment. It not only shows high yield (grain and biomass) and good quality, but also displays excellent characteristics of strong tillering ability, good biotic and abiotic stress resistances, and high grain protein content (Kuleung *et al.* 2004; Oettler *et al.* 2005; Audenaert *et al.* 2014). During the past few decades, hexaploid triticale has been widely cultivated for grain production. Currently, hexaploid triticale is an important forage crop and a potential energy plant (Cantale *et al.* 2016; Kang *et al.* 2016). So far, there have been few reports on any successful genetic transformation systems for rye and hexaploid triticale. Reports have indicated that transgenic rye plants were obtained by the *Agrobacterium*-mediated method with an efficiency of only 1–4% (Popelka and Altpeter 2003), and marker-free transgenic rye plants were produced by the biolistic-mediated method in low copy numbers (Popelka *et al.* 2003). Transgenic plants of triticale *cv*. Wanad were produced by the *Agrobacterium*-mediated method with efficiency varying from 0 to 16% (Nadolska-Orczyk *et al.*

2005). Such inefficient transformation systems would hinder gene editing-based genetic modifications in these two plants, so the efficiency needs to be improved.

Current studies have shown that genes encoding some developmental regulators or transcription factors (TFs) can be used to improve plant regeneration and transformation efficiencies, which can facilitate the establishment of transformation or gene editing systems in several recalcitrant plant species. For example, by overexpressing the maize-derived regeneration-related genes *BBM* and *WUS2* in maize, *indica* rice, sugarcane and sorghum, their transformation efficiencies were massively improved by several folds (Lowe *et al.* 2016; Mookkan *et al.* 2017). Recently, transgenic plants have been produced from the mature embryos and young leaf base tissues of many cereal plants when *BBM* and *WUS2* were applied in the transformation experiments (Lowe *et al.* 2016; Wang *et al.* 2023). Transgenic plants were efficiently obtained from a few genotypes of common wheat and durum wheat after the expression vector containing the *GRF-GIF* chimeric gene was used in the transformation (Debernardi *et al.* 2020). Recently, two DNA binding with one finger (DOF) TFs, *TaDOF5.6* (*TraesCS6A02G274000*) and *TaDOF3.4* (*TraesCS2B02G592600*), were found to greatly enhance the transformation efficiencies of different wheat varieties (Liu *et al.* 2023).

Specifically, the WOX5 member of the WUS family of homeodomain transcription factors has been found to act as a regulator of stem cell maintenance in plants (van der Graaff *et al.* 2009; Forzani *et al.* 2014). This protein is specifically expressed in the quiescent center (QC), which promotes stem cell fate and regulates QC proliferation through repressing the *CYCLIN D* activity (Forzani *et al.* 2014). In *Arabidopsis* root meristem, WOX5 moves to the columella stem cells (CSCs) from the QC and represses the transcription factor gene *CYCLING DOF FACTOR 4* (*CDF4*) to control stem cell fate (Pi *et al.* 2015). In *Arabidopsis*, the WOX11/12 proteins bind directly to the promoters of *WOX5/7* to boost root primordia initiation and organogenesis (Hu and Xu 2016). Studies have shown that WOX5 is associated with organ regeneration by promoting auxin production and enhancing cytokinin sensitivity, which is required for pluripotency acquisition (Zhai and Xu 2021). To date, only one study in plants reported that the overexpression of the wheat gene *TaWOX5* dramatically improved wheat transformation efficiency with less genotype dependence (Wang *et al.* 2022).

Considering the dramatic roles of plant developmental regulators in regeneration and transformation in a few key crops like maize, wheat, rice, sorghum, and sugarcane, in this study we established efficient *Agrobacterium*-mediated transformation systems for cultivated *T. monococcum*, hexaploid triticale and rye using their immature embryos through the application of the *TaWOX5* gene. These new systems can facilitate the improvement of these three species as well as functional genomics studies of wheat.

2. Materials and methods

2.1. Plant materials and growth conditions

The *T. monococcum* accession PI428182 was kindly provided by Dr. Xiaohui Li at Capital Normal University, Beijing, China. Three hexaploid triticale advanced lines (ZS3297, ZS1257, and ZS3224), which were developed using the sterile triticale material containing the *TaMs2* gene from wheat *via* recurrent selection breeding strategy, were kindly provided by Prof. Zengyuan Wang at the Institute of Crop Sciences of Chinese Academy of Agricultural Sciences. Another hexaploid triticale genotype, Lin456, which was developed through the hybridization of an octoploid triticale line H400 (AABBDDRR) and a wheat line Lin56 (AABBDD) followed by consecutive self-crossing, was kindly provided by Prof. Fangpu Han at the Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences. A *Secale* cereale landrace Lanzhou Heimai was maintained in our laboratory. The materials of *T. monococcum* and hexaploid triticale used in this study were of the winter type. They were planted at the end of October in 2015 in a field in Beijing and transplanted into pots (30 cm in diameter and 25 cm in height) after vernalization in early December 2015. Lanzhou Heimai was directly sown in pots. All the plants in pots were maintained in a growth chamber at 25°C/20°C (day/night), 16 h/8 h (light/dark) with 300 μmol m^{-2} s^{-1} light intensity and 45% humidity, which were automatically controlled for collecting the immature grains for genetic transformation. Aphids were controlled with sticky colored cards (Zhengzhou Oukeqi Instruments Co., Ltd., China) and powdery mildew (PM) was controlled by the application of triadimefon (Jinan Luba Pesticides Co., Ltd., China).

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2.2. Plasmids and bacterial strain

All the expression vectors used in this study, with or without the *TaWOX5* gene (MN412514.1) located on wheat chromosome 3D, were previously constructed in our laboratory (Wang *et al.* 2017, 2022). The vector *pWMB110-GUS* contains the *β-glucuronidase* (*GUS*) and *bialaphos resistance* (*bar*) genes, and *pWMB111-TaWOX5* contains the *TaWOX5* and *bar* genes, of which *bar*, *GUS*, and *TaWOX5* were employed as the selection marker, reporter marker, and regeneration promotor, respectively. The two vectors were maintained in *Agrobacterium* strain C58C1 for the genetic transformation of the plant materials mentioned above.

2.3. Plant transformation

Immature grains of *T. monococcum*, hexaploid triticale and rye were collected at 14–15 days post anthesis (DPA), sterilized with 75% ethanol for 1 min and 15% sodium hypochlorite solution for 5 min, and then rinsed five times with sterile water. The immature embryos were isolated from the sterilized grains and infected by *Agrobacterium* as described in a published protocol (Wang *et al.* 2022).

Briefly, the isolated immature embryos from the three wheat-related species were collected in a 2.5-mL Eppendorf tube containing 2 mL WLS liquid medium (1/10 Linsmaier and Skoog (LS) salts, 1/10 Murashige and Skoog (MS) vitamins, 10 g L^{-1} glucose, 0.5 g L^{-1} 2-(N-morpholino) ethanesulfonic acid (MES) and 100 μmol L^{-1} acetosyringone (AS), pH 5.8) , centrifuged at 4°C for 10 min at 7,500 r min^{-1}, and incubated in the same volume of *Agrobacterium* solution for 10 min at room temperature after pipetting off the WLS liquid medium. The *Agrobacterium*-infected immature embryos were placed on co-cultivation medium (WLS liquid medium plus 0.85 mg L^{-1} $AgNO_3$, 1.25 mg L^{-1} $CuSO_4{\cdot}5H_2O$ and 8 g L^{-1} agarose) with the scutellum facing upwards at 23°C in darkness. Two days later, the embryonic axes of the co-cultivated immature embryos were removed with a scalpel and the remaining scutella were cultured on resting medium (LS salts, MS vitamins, 0.5 mg L^{-1} 2,4-D, 2.2 mg L^{-1} picloram, 0.85 mg L^{-1} $AgNO_3$, 100 mg L^{-1} ascorbic acid, 250 mg L^{-1} carbenicillin, 100 mg L^{-1} cefotaxime, 1.95 g L^{-1} MES, and 5 g L^{-1} agarose) at 25°C for 5 days in darkness. Next, the tissues were transferred onto selection medium (resting medium plus 5 mg L^{-1} phosphinothricin (PPT, Sigma, no. 45520)) and cultured for 2 weeks. Afterwards, each callus was divided into two parts that were cultured on induction medium containing 10 mg L^{-1} PPT for 3 weeks. Finally, embryonic calli were placed onto differentiation medium containing 5 mg L^{-1} PPT at 25°C with a daily interval of 100 μmol m^{-2} s^{-1} light for 14 h and dark for 10 h. At the appropriate time, regenerated green shoots were transferred onto rooting medium containing 5 mg L^{-1} PPT. The candidate plantlets with well-developed leaves and roots were transplanted into pots and cultivated in the growth chamber described above.

To compare the functions of *TaWOX5* in the transformation of the three wheat-related species, two types of transformation experiments for each genotype of each plant species were designed. In the first type, the two vectors *pWMB110-GUS* and *pWMB111-TaWOX5* were co-transformed into the immature embryos of the three plant species by mixing the *Agrobacterium* cultures. In second type, only the vector *pWMB110-GUS* was transformed into the immature embryos with its corresponding *Agrobacterium* as a control. In each transformation experiment, three biological replicates were set up.

2.4. DNA extraction and polymerase chain reaction analysis

Genomic DNA was extracted from the leaves of the T_0 and T_1 transgenic plants using the Nuclear Plant Genomic DNA Kit (CW Bio Inc., China). After the final centrifugation for DNA cleaning, 50 μL sterile water was added to the tube to dissolve the DNA. Two pairs of primers, 5´-CAAGGAAATCCGCAACCATATC-3´ and 5´-TCAAACGTCCGAATCTTCTCCC-3´ specific to the *GUS* gene, and 5´-ACCATCGTCAACCACTACATCG-3´ and 5´-GCTGCCAGAAACCACGTCATG-3´ specific to the *bar* gene, were employed to detect the presence of the transgenes in T_0 and T_1 plants by polymerase chain reaction (PCR). Each 20 μL PCR reaction system included 1 μL template DNA (100 ng μL^{-1}), 1 μL forward primer (10 μmol L^{-1}), 1 μL reverse primer (10 μmol L^{-1}), 10 μL 2× *Taq* Master Mix (containing Mg^{2+} and dNTP, Vazyme Biotech Co., Ltd., China) and 7 μL ddH_2O. The PCR was run in a T100™ thermal cycler (BIO-RAD, California, USA) with a program of an initial denaturation at 95°C for 5 min, 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C for amplification, and a final extension at 72°C for 8 min. The PCR products were separated on a 1.5% agarose gel and visualized by Genecolour DNA Staining™ II (Gene-Bio Co., Ltd., Beijing, China).

2.5. Histochemical staining and QuickStix strip detection

Expression of the GUS protein can be visually observed through the color change of the stained tissues by a histochemical staining assay. Specifically, leaf pieces from T_0 and T_1 transgenic plants were immersed in 2 mL tubes with histochemical staining solution (0.1 mol L^{-1} $NaPO_4$ buffer (pH 7.0), 10 mmol L^{-1} Na_2-EDTA, 0.2 mmol L^{-1} ferricyanide, 0.2 mmol L^{-1} ferrocyanide, 0.8 g L^{-1} 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc), 20% methanol (v/v), and 0.5% Triton-100 (w/v)) at 37°C for 12–24 h. Then, the stained tissues were decolorized with 70% ethanol at room temperature. Additionally, transgenic plants were also assessed for expression of the *bar* gene using a QuickStix Kit (Envirologix, USA), in which the leaves were sampled from T_0 and T_1 transgenic plants, ground with a small plastic pestle in a 1.5-mL tube with 0.5 mL buffer, and the special band could be checked after a QuickStix strip was put into the tube for 5 min.

2.6. Fluorescence *in situ* hybridization and genomic *in situ* hybridization analysis

Firstly, fresh roots of 1–2 cm in length were collected from each germinated seed of the transgenic plants at 25°C in a 0.2 mL tube and treated with nitrous oxide for 2 h. Next, 0.1 mL glacial acetic acid (90%, v/v) was added to the tube for 5 min to fix the chromosomal morphology. After rinsing with sterile water, the root tips were cut off and digested in enzymatic hydrolysate for 1 h and chromosome slides were prepared. Genomic *in situ* hybridization (GISH) was performed to visualize the rye chromosomes in hexaploid triticale transgenic plants according to the description in a previous protocol (Han *et al.* 2006), in which the genomic DNA of rye as a probe was labeled using the DIG-Nick Translation Mix Kit (Roche, Germany). Fluorescence *in situ* hybridization (FISH) was performed to detect the transgenes in transgenic plants, in which the *GUS* gene was used as a probe labeled with the fluorescein isothiocyanate conjugated Anti-Digoxigenin Fluorescein Fab Fragments Kit (Roche, Germany) (Liu *et al.* 2020). The hybridization procedures were followed as previously described (Yuan *et al.* 2015). Chromosomes and hybridization signals were observed under a fluorescence microscope (BX51, Olympus, Japan).

2.7. Statistical analysis

Data analysis was performed through SPSS software, and $P \leq 0.05$ was considered as representing statistical significance.

3. Results

3.1. Generation of transgenic plants in the three plant species by the application of gene *TaWOX5*

In total, 273 immature embryos of *T. monococcum* genotype PI428182; 168, 170, 164 and 199 immature embryos of hexaploid triticale genotypes Lin456, ZS3297, ZS1257 and ZS3224; and 230 immature embryos of rye genotype Lanzhou Heimai were either co-transformed with the vectors containing *GUS* and *TaWOX5* genes or single-transformed with the vector containing *GUS* gene as the control treatment, mediated by *Agrobacterium* at least three biological replications (Table 1). After callus induction culture on selection medium, embryonic calli were produced in the co-transformation experiments (Fig. 1-A, D, and G), but no embryonic calli were obtained in the experiments with control vector only. When all were cultured on differentiation medium for one week, the embryonic calli in the co-transformation experiments generated many green shoots (Fig. 1-B, E, and H), but no shoots were produced in the control experiments. Ultimately, 258, 222 and 18 transgenic plants were obtained from *T. monococcum*, hexaploid triticale, and rye, respectively (Fig. 1-C, F, and I; Table 1).

3.2. Detection of transgenic plants by PCR, QuickStix strip and histochemical staining

All the candidate transgenic plants obtained from the

Table 1 Overexpression of *TaWOX5* increases the transformation efficiency of the three species related to wheat

Genotype	Species	Control vector				*TaWOX5* vector			
		Experiments	Immature embryos transformed	Positive plants	Transformation efficiency (%)	Experiments	Immature embryos transformed	Positive plants	Transformation efficiency (%)
PI428182	*Triticum monococcum*	3	198	0	0	3	273	258	94.4±0.3
Lin456	Hexaploid triticale	3	203	0	0	3	168	88	52.1±0.6
ZS3297	Hexaploid triticale	3	183	0	0	3	170	70	41.1±0.7
ZS1257	Hexaploid triticale	3	150	0	0	3	164	32	19.4±0.4
ZS3224	Hexaploid triticale	3	213	0	0	3	199	32	16.0±0.3
Lanzhou Heimai	*Secale cereale*	3	196	0	0	3	230	18	7.8±0.2

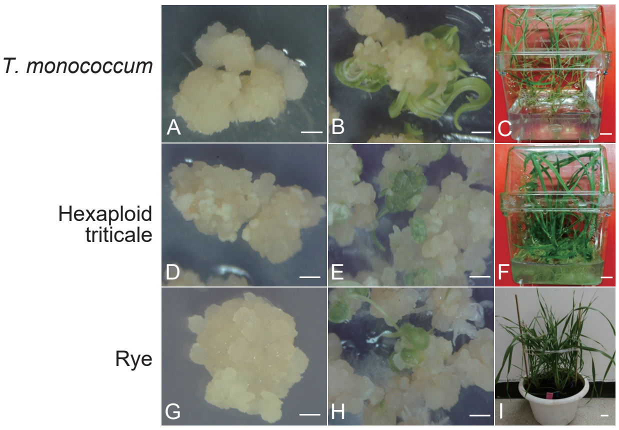

Fig. 1 Callus induction and shoot regeneration from the transformed immature embryos of *Triticum monococcum*, hexaploid triticale, and rye mediated by *Agrobacterium*. A, D and G, callus induction. B, E and H, green shoot differentiation. C and F, elongation of candidate transgenic plants. I, transplanting of candidate transgenic plants. Scale bar=5 mm.

three plant species were assessed for the existence of the *GUS* and *bar* genes by PCR, and the expression of the *bar* gene by QuickStix strip and GUS activity by histochemical staining. The results showed that a fragment of 995 bp in length specific to the *GUS* gene and a fragment of 500 bp in length specific to the *bar* gene were amplified from the transgenic plants (Fig. 2-A–F). The special band associated with the bar protein was detected in the transgenic plants, and the blue signals associated with GUS protein were clearly seen in the leaf pieces of the transgenic plants (Fig. 2-G–J). These results indicated that the two exogenous genes were successfully integrated and stably expressed in the genomes of *T. monococcum*, hexaploid triticale, and rye. The transformation efficiencies were 94.4% for *T. monococcum* genotype PI428182; 52.1, 41.2, 19.4, and 16.0% for hexaploid triticale genotypes Lin456, ZS3297, ZS1257, and ZS3224, respectively; and 7.8% for rye genotype Lanzhou Heimai (Table 1).

3.3. Analysis of the transgenic plants by GISH and FISH

The seeds of T_0 were harvested from the T_0 transgenic plants of *T. monococcum* and hexaploid triticale. However, no seeds were harvested from the T_0 transgenic plants of rye because rye is a cross pollinated

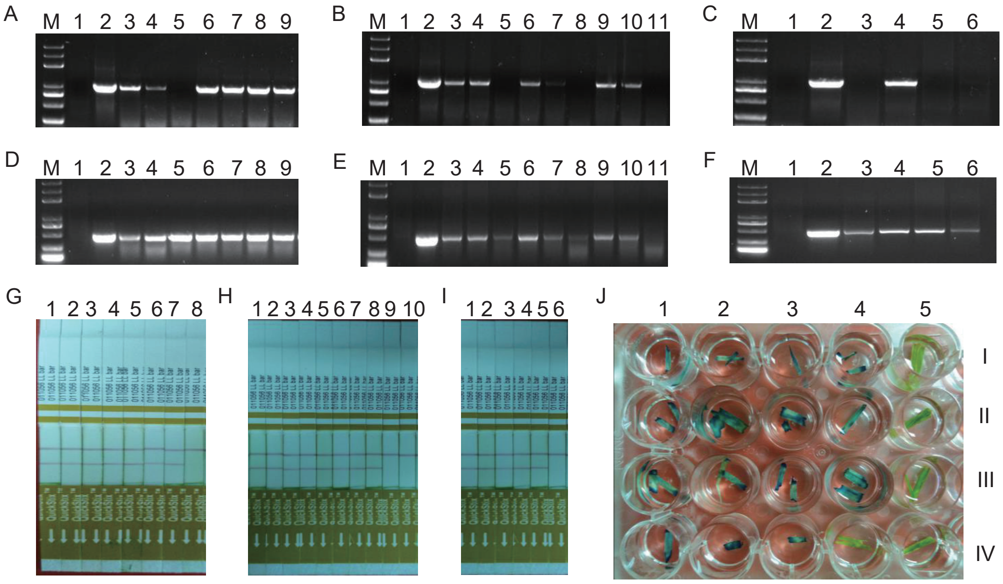

Fig. 2 Detection of the *GUS* and *bar* genes in T_0 putative transgenic plants of the three wheat-related species by polymerase chain reaction (PCR), QuickStix strip, and histochemical staining. A–C, detection of the *GUS* gene in *Triticum monococcum*, triticale, and rye putative transgenic plants by PCR, respectively. D–F, detection of the *bar* gene in *T. monococcum*, hexaploid triticale, and rye putative transgenic plants by PCR, respectively. M, 5 kb DNA marker; 1, wild type (WT); 2, positive control; 3–9 in A and D, putative transgenic plants of *T. monococcum*; 3–11 in B and E, putative transgenic plants of hexaploid triticale; 3–6 in C and F, putative transgenic plants of rye. G–I, detection of the *bar* gene in *T. monococcum*, hexaploid triticale and rye putative transgenic plants by QuickStix strip, respectively. G-8, H-10, and I-6 represent WT respectively. J, detection of the *GUS* gene in *T. monococcum*, hexaploid triticale and rye putative transgenic plants by histochemical staining. I1–I4, leaf samples were collected from the *T. monococcum* transgenic plants; I5, WT of *T. monococcum*; II1–III4, leaf samples collected from hexaploid triticale transgenic plants; III5, WT of triticale; IV1–IV4, leaf samples collected from rye transgenic plants; IV5, WT of rye.

crop and no seeds were available under the controlled environmental conditions. The integration sites and copy numbers of the *GUS* gene in the transgenic plants of *T. monococcum* and hexaploid triticale were identified by FISH and GISH using their root tips sampled from germinated T_1 seeds. In the randomly picked *T. monococcum* transgenic lines identified by FISH, a pair of chromosomes with green signals for the *GUS* gene were clearly observed, indicating that the foreign gene was integrated into the genome as a single copy at the distal or near-distal region of the chromosome (Fig. 3-A–C). In the randomly picked hexaploid triticale transgenic lines identified by combined GISH and FISH, some lines were heterozygous at the integration site having one chromosome with a signal (Fig. 3-D), and some lines were homozygous at the integration site having a pair of chromosomes with signals (Fig. 3-E). Thus, the *GUS* gene was inserted into the genome of the transgenic plants as a single copy at the distal or near-centromere region of the chromosomes. Furthermore, fluorescence signals were observed on the chromosomes of durum showing a dark or light blue color, and the chromosomes of rye showing a red color, indicating that the transgene was randomly integrated into the AABB and RR genomes in hexaploid triticale (Fig. 3).

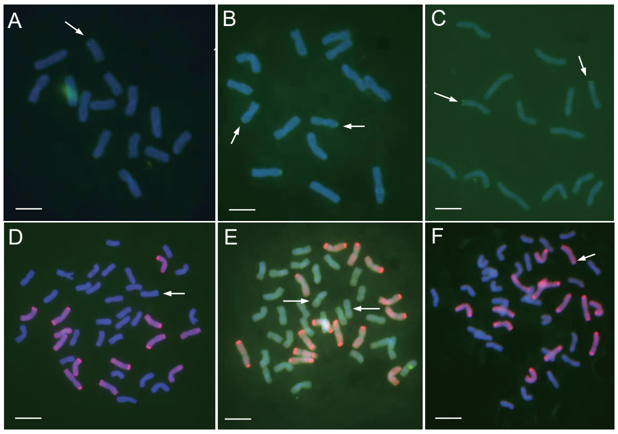

Fig. 3 Integration of the *GUS* genes into the genome of transgenic *Triticum monococcum* and hexaploid triticale plants identified by fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH). A, identification of the NT1-1 transgenic line of *T. monococcum* by FISH. B, identification of the NT1-4 transgenic line of *T. monococcum* by FISH. C, identification of the NT2-17 transgenic line of *T. monococcum* by FISH. D, identification of the NT10–14 transgenic line of hexaploid triticale by combined FISH and GISH. E, identification of the NT6-1 transgenic line of triticale by combined FISH and GISH. F, identification of the NT19-4 transgenic line of triticale by combined FISH and GISH. The integration locus of the *GUS* gene is indicated by a white arrow. Scale bar=10 µm.

3.4. Inheritance analysis of the transgene in T_1 transgenic plants of *T. monococcum* and hexaploid triticale

Three transgenic lines (NT1–NT5 from PI428182) of *T. monococcum*, and 12 transgenic lines (NT6–NT10 from Lin456, NT11–NT15 from ZS3297, NT16–NT20 from ZS1257, and NT21–NT25 from ZS3224) of hexaploid triticale in the T_1 generation were used to detect the *GUS* gene by PCR and histochemical staining for segregation analysis (Table 2). The detection results by the two methods were quite consistent with each other. The chi-square analysis demonstrated that the *GUS* gene was inherited in the T_1 generation at a 3:1 segregation ratio (Table 2), indicating that the transgene in the T_0 generation was always heterozygous. Based on the *P*-value of the segregation ratio, the inheritance of *GUS* in our transgenic plant events almost fitted to Mendel's law (Table 2), and this conclusion was also supported by the FISH results, in which the target gene was integrated in the genome of the tested transgenic plants as a single copy (Fig. 3). Our results also proved that the transgene

Table 2 Segregation analysis of the *GUS* gene in T_1 *Triticum monococcum* and hexaploid triticale transgenic plants

Line	Genotype	Species	Total plants detected	Histochemical stain			
				GUS$^+$	*GUS*$^-$	χ^2	*P*-value
NT1	PI428182	*Triticum monococcum*	20	16	4	0.066	0.796
NT2			21	15	6	0.015	0.899
NT3			18	14	4	0.000	1.000
NT4			87	60	27	1.383	0.240
NT5			60	42	18	0.556	0.456
NT6	Lin456	Hexaploid triticale	19	15	4	0.017	0.894
NT7			20	17	3	0.600	0.438
NT8			18	14	4	0.000	1.000
NT9			40	28	12	0.300	0.584
NT10			62	45	17	0.086	0.769
NT11	ZS3297	Hexaploid triticale	19	16	3	0.438	0.507
NT12			20	15	5	0.066	0.796
NT13			21	16	5	0.015	0.899
NT14			32	20	12	2.042	0.153
NT15			46	38	8	1.043	0.307
NT16	ZS1257	Hexaploid triticale	17	13	4	0.019	0.888
NT17			19	15	4	0.017	0.894
NT18			18	13	5	0.000	1.000
NT19			39	30	9	0.009	0.926
NT20			52	40	12	0.026	0.873
NT21	ZS3224	Hexaploid triticale	20	14	6	0.066	0.796
NT22			20	16	4	0.066	0.796
NT23			18	13	5	0.000	1.000
NT24			28	16	8	1.000	0.317
NT25			49	38	11	0.061	0.805

could be stably inherited in transgenic *T. monococcum* and hexaploid triticale plants.

4. Discussion

4.1. Main factors affecting successful transformation in triticeae plants

Although transgenic plants have been successfully produced and reported in many cereal species, producing transgenic events is still a bottleneck for the species related to wheat. When the immature embryos of einkorn wheat in *T. monococcum* were transformed using biolistic-mediated transformation, the transformation efficiency was very low (Miroshnichenko *et al.* 2018). In an *Agrobacterium*-mediated transformation report in rye, the efficiency varied from 1 to 4% (Popelka and Altpeter 2003). In triticale, the transformation efficiency of a genotype Wanad ranged from 0.00 to 6.79% using an *Agrobacterium*-mediated transformation strategy (Bińka *et al.* 2012). In our present study on the *Agrobacterium*-mediated transformation of *T. monococcum*, hexaploid triticale, and rye, when assisted with *TaWOX5* and using their immature embryos, the efficiencies were 94.4% for *T. monococcum* accession PI428182; 52.1, 41.2, 19.4, and 16.0% for hexaploid triticale Lin456, ZS3297, ZS1257, and ZS3224, respectively; and 7.8% for rye cultivar Lanzhou Heimai (Table 1).

The immature embryo of cereal plants is the most widely used explant for genetic transformation because of its strong regeneration ability (Lowe *et al.* 2016; Wang *et al.* 2017, 2022). In our present work, we also chose the immature embryos of *T. monococcum*, hexaploid triticale, and rye as explants to generate the transgenic plants. However, the physiological status of an immature embryo is greatly restricted by environment conditions (especially temperature), pests and diseases, which severely impacts the regeneration potential and transformation efficiency. The recipient materials must be grown in suitable growth environments. In particular, the temperature is a key environmental factor, and high temperatures should be avoided before harvest of the immature embryos (Wang *et al.* 2014). One recommendation is to use materials with a strong regeneration ability and disease resistance as the recipients in transformation (Liang *et al.* 2022).

4.2. Overexpression of transcriptional factors can improve plant transformation efficiency

For the plant species or genotypes recalcitrant to *in vitro* regeneration, overexpression of plant developmental regulators can improve their regeneration ability and transformation efficiency (Chen *et al.* 2022). The wheat gene *TaWOX5* was shown to be effective in overcoming genotype dependence in wheat genetic transformation in our previous study (Wang *et al.* 2022). In fact, the native *TaWOX5* was specifically expressed in wheat root or callus induced by auxin and cytokinin, indicating that *TaWOX5* is related to the hormonal regulation in somatic embryogenesis in plants (Zhao *et al.* 2014). In legumes, *WOX5* is involved in the development of the nodule meristem and expressed at different nodule formation stages (Osipova *et al.* 2011). In *Medicago truncatula*, *MtWOX5* is expressed in auxin-induced root primordia and root meristems and might have a role in pluripotent stem cell induction (Chen *et al.* 2009). In our present study, by overexpressing *TaWOX5*, transgenic plants were efficiently produced from the *Agrobacterium*-infected immature embryos of *T. monococcum*, hexaploid triticale and rye with optimal transformation efficiencies. To further enhance the transformation efficiencies of these three plant species, we will assess the roles of the reported regeneration-associated genes *BBM*, *WUS2*, *GRF-GIF*, *TaDOF5.6* and *TaDOF3.4*, and make extra efforts to identify other developmental regulator-encoding genes related to plant regeneration in our future work.

4.3. Application of FISH technology in identifying transgenic plants

In this study, we successfully detected the presence of the *GUS* and *bar* genes in the transgenic plants firstly by PCR, QuickStix strip, and histochemical staining (Fig. 2). The PCR results indicated that all the transgenic plants contained the *bar* gene, and some of the transgenic plants contained the *GUS* and *bar* genes (Fig. 2-A–F). This is because the two vectors *pWMB110-GUS* (carrying *GUS* and *bar* genes) and *pWMB111-TaWOX5* (carrying *TaWOX5* and *bar* genes) were co-transformed in our transformation experiments. Thus, the transgenic plants with two vectors or either one alone harbored *bar*, while only the transgenic plants with *pWMB110-GUS* harbored *GUS*, which resulted in the inconsistent detection results for the *bar* and *GUS* genes by PCR analysis.

FISH was also used to detect transgenic plants, and it can clearly locate the physical position of a transgene integrated into a chromosome of the transgenic plant. FISH analysis has proven to be a solid tool for determining copy number and anchoring the integration site of a target gene on the chromosomes (Harrison and Heslop-Harrison 1995). According to the published reports using FISH, transgenes are mostly integrated into the distal regions of chromosomes in the transgenic plants of oat (Svitashev and Somers 2002), tobacco (Iglesias *et al.* 1997), barely (Salvo-Garrido *et al.* 2001), and wheat (Liu *et al.* 2020). One study reported that the transgene did not show pronounced preferences for integration at the distal chromosomal sites in transgenic wheat plants (Abranches *et al.* 2000). Here, we detected the transgenic lines of *T. monococcum* and hexaploid triticale containing the T-DNA using the vector carrying *GUS* and *bar* as a probe for clarifying the copy number and chromosomal location of the transgene. We found that the foreign gene was mostly integrated at the distal regions of the chromosomes in *T. monococcum* and hexaploid triticale, and integrated into the region near the centromere of the chromosome in a few hexaploid triticale transgenic plants. Our findings by FISH are mostly consistent with other reported results (Iglesias *et al.* 1997; Abranches *et al.* 2000; Salvo-Garrido *et al.* 2001; Svitashev and Somers 2002; Liu *et al.* 2020). Theoretically, the distal regions of chromosomes contain less heterochromatin, where chromosomes more frequently break, and the T-DNA expression cassette is more easily integrated compared to the centromere region (Svitashev and Somers 2002). In addition, in the 20 hexaploid triticale transgenic lines identified by FISH, the T-DNA was found to be integrated onto the RR genome in one line. This indicated that the foreign DNA fragment was randomly integrated into the AABB and RR genomes in hexaploid triticale. According to the results by FISH, the target gene was detected in a pair of chromosomes of the transgenic *T. monococcum* and hexaploid triticale plants, which meant that it was integrated into the transgenic plants as a single copy (Fig. 3). Therefore, the inheritance of the target gene in T_1 plants followed Mendel's law (Table 2), and these two results were consistent with each other.

Unfortunately, we were not able to characterize the rye transgenic plants by FISH or to perform the genetic analysis of the transgene in rye transgenic plants in this study, simply because we failed to obtain T_1 seeds from the T_0 plants. To our knowledge, rye is a cross pollinated crop, and its seed-setting rate in self-crossing is less than 10%. However, we cultivated the rye transgenic plants in an environment-controlled growth chamber, so the conditions were not desirable for their pollination. In future work, we need to improve the environmental conditions in the growth chamber and make crosses artificially between different rye transgenic plants to produce seeds for further research.

4.4. Potential roles of efficient transformation systems for the species related to wheat

In recent years, CRISPR/Cas9 technology has greatly accelerated the progress of molecular breeding in crops. With the improvement of transformation efficiencies in species related to wheat and the availability of more sequenced genome data in wheat, more wheat genes will be easily identified functionally in the diploid relatives of wheat such as *T. monococcum* and rye. We anticipate that our improved transformation efficiency will accelerate functional genomic studies in the species related to wheat by transgenic and gene editing approaches.

5. Conclusion

We efficiently generated transgenic plants of *T. monococcum*, hexaploid triticale, and rye using their immature embryos by *Agrobacterium*-mediated transformation, which was assisted with the regeneration-related wheat gene *TaWOX5*. The transformation efficiencies for the three plant species were 94.4, 32.2 (16.0–52.1), and 7.8%, respectively. The FISH and GISH analyses confirmed the frequent integration of the transgene at the proximal regions of chromosomes without genome priority and mostly as a single copy. Genetic analysis revealed that the transgene could be stably inherited in the T_1 generation by Mendel's law.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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