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Oligonucleotide-directed gene repair in wheat using a transient plasmid gene repair assay system

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Abstract Oligonucleotide-directed gene repair is a potential technique for agricultural trait modification in economically important crops. However, large variation in the repair frequencies among the scientific reports indicates that there are many factors influencing the repair process. We report here a transient assay system using GFP as a reporter for testing the efficiency of plasmid DNA repair in cultured wheat cells. This assay showed that osmotic medium supplemented with 2,4-D increased the oligo-targeting frequency, and that the repair of a point mutation was more efficient than repair of a single base deletion mutation in cultured scutellum cells of immature wheat embryos. This study provides the first evidence that oligonucleotide-directed mutagenesis is applicable to regenerable cultured wheat scutellum cells.

Keywords Gene repair · GFP · Immature embryo · Plasmid DNA · Single-stranded DNA oligonucleotide · Wheat

Abbreviations *2,4-D*: 2,4-dichlorophenoxyacetic acid · *GFP*: green fluorescent protein · *RDO*: RNA/DNA oligonucleotide · *SDO*: single-stranded DNA oligonucleotide

Introduction

The successful site-specific modification of plasmid and chromosomal genes by chimeric RNA/DNA oligonucleotides (RDO) was first reported in mammalian cell lines in 1996 (Cole-Strauss et al. 1996; Yoon et al. 1996). Since then, some successful applications of this technique in mammalian cells and animal models were achieved both in vivo and in vitro (Kren et al. 1997; Alexeev and Yoon 1998; Alexeev et al. 2000; Bartlett et al. 2000; see review Igoucheva et al. 2004). Studies using nuclear extracts, cultured bacterial, yeast and human cells have yielded some insights into the mechanism(s) and factors involved in this process. However, the exact mechanism of oligonucleotide-directed gene conversion is still elusive. It may involve two steps: homologous pairing that results in the formation of a D-loop structure, and subsequent endogenous DNA repair activity (Gamper et al. 2000a,b; Drury and Kmiec 2003). Some DNA repair proteins and homologous pairing proteins, such as MSH2, RAD51 and RAD54, are involved in chimeric oligonucleotide-directed gene repair (Cole-Strauss et al. 1999; Liu et al. 2002a, 2004; Igoucheva et al. 2002). The DNA strand of the chimeric oligonucleotide is postulated to be used by the endogenous repair machinery as a template for correction (Gamper et al. 2000a,b; Igoucheva et al. 2001; Liu et al. 2001). This finding and early studies using single-stranded oligodeoxynucleotides for site-specific alteration of targeted DNA in mammalian cells and yeast (Moerschell et al. 1988; Yamamoto et al. 1992) prompted the development of chemically modified single-stranded DNA oligonucleotides (SDO) as the targeting molecule. SDOs showed similar gene repair frequency, but more reproducible results, to chimeric oligonucleotides (Liu et al. 2001; Igoucheva et al. 2001; Parekh-Olmedo et al. 2002; Alexeev et al. 2002). The mechanism involved in SDO-directed gene repair may differ from RDO-directed gene repair, for example some studies reported that MSH2 does not participate in or even inhibits SDO-directed gene repair (Gamper et al. 2000b; Dekker et al. 2003), while it promotes RDO-directed gene repair (Cole-Strauss et al.

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1999). Also, the intermediates formed by SDO and by RDO are different in their structure and stability (Igoucheva et al. 2002, 2004). Cellular activities such as the transcription and replication state of the targeted gene, and the availability of the homologous pairing and gene repair enzymes play an important role in the oligonucleotide-directed gene repair (Liu et al. 2002b; Igoucheva et al. 2002, 2003; Brachman and Kmiec, 2004). Successful gene correction will depend on the right targeting molecule being exposed at the right time and right location to the target DNA in a biologically active cell. Since it is an extremely complex process, cells competent to the oligonucleotide-directed gene repair will be a small portion in a given cell population. Some conflicting results in the literature reflect this complexity (see review Igoucheva et al. 2004).

In plants, the first successful application of chimeric oligonucleotide-directed gene conversion was achieved in tobacco (Beetham et al. 1999) and maize (Zhu et al. 1999). In both cases, the acetolactate synthase/acetohydroxyacid synthase gene (*ALS/AHAS*) and a transgene (a nonfunctional green fluorescent protein gene) were modified by targeting chimeric oligonucleotides. The resulting herbicide-resistant maize plant exhibited segregation expected at a single locus (Zhu et al. 2000), indicating this mutation was stably inherited. Similar gene targeting was reported recently in tobacco and rice (Kochevenko and Willmitzer 2003; Okuzaki and Toriyama 2004). Oligonucleotide-directed gene repair using an in vitro assay system ("cell-free" extracts) was also reported in cultured cells of tobacco and banana, as well as maize embryo and spinach chloroplast (Rice et al. 2000; Kmiec et al. 2001).

Although the technique shows great potential for human medicine, agriculture and functional genomics (Hohn and Puchta 1999; Rice et al. 2001), the repair frequency is reported to be inconsistent ranging from a nondetectable frequency (van der Steege et al. 2001; Ruiter et al. 2003) to as high as 40% (Cole-Strauss et al. 1996; Kren et al. 1997). Ruiter et al. (2003) reported that the spontaneous mutation frequency in both tobacco and oilseed rape cells obscures the effect of chimeric oligonucleotide-directed gene repair. In their experiments, the frequency of oligonucleotide-directed gene repair induced mutations of a chromosomal gene was not higher than that of the spontaneous mutation rate at approximately once in every 2×10^7 . However, other reports on tobacco cells (Beetham et al. 1999; Kochevenko and Willmitzer 2003) showed 10–20 times higher frequency of oligonucleotide-induced mutation than spontaneous mutation. These variations indicate that different cell lines, different physiological stages of the cells, different selection pressures and even different culture conditions may affect the gene repair ability of the cell. As oligonucleotide-directed gene targeting involves many endogenous pathways including homologous pairing, mismatch recognition and DNA repair (Igoucheva et al. 2004), the biological activity of the targeted cells is important for successful targeting.

To test the utility of oligonucleotide-directed gene repair in wheat, we developed an assay system in which a non-functional plasmid Green Fluorescent Protein (GFP) gene

could be repaired by oligonucleotide-mediated gene targeting in wheat scutellum cells. The restoration of green fluorescence in the cell is a visual marker indicating the targeted gene repair event(s).

Materials and methods

Oligonucleotides

A chemically modified single-stranded oligonucleotide named SDO^{GFP} (Fig. 1a), 5'-CCACCATGGTGAGC AAGGGCGAGGAGCTGTTCACCGGGGTG-3' with 5'-end labeled with Cy3 and 3'-end with inverted cytosine base; and a chimeric RNA/DNA oligonucleotide (for details, see Beetham et al. 1999) named RDO^{GFP} were used for correcting the mutation in a GFP gene.

Reporter plasmid construction

The pMON30049 vector encodes a synthetic green fluorescent protein (GFP) gene with improved GFP expression in monocots and enhanced brightness of green fluorescence, under the control of the enhanced cauliflower mosaic virus 35S promoter (Pang et al. 1996). This vector was modified by PCR amplification using primers designed to modify the beginning of the coding region to match the sequence of the oligonucleotide SDO^{GFP} and RDO^{GFP}. The PCR product was then cloned into the *EcoRI* and *BamHI* sites of the pMON30049 to replace the original GFP gene. The resulting construct was renamed pGFP. The activity of pGFP in the living plant cells was confirmed by transient expression of GFP in the wheat scutellum tissue by bombardment. Primers used in PCR were GFP-up1 (5'-AAGGGCGAGGAGCTGTTCACCTGGCG-3'), GFP-up2 (5'-CCC GGATCCACCATGGTGAGCAAGGGCGAGGA GCTGTTCA-3') and GFP-low (5'-CCGGAATTCAGATCTTCACTTGTAGA-3'). PCR was performed using *Pfx* Polymerase (Invitrogen) and buffers provided by the manufacturer with primers GFP-up1 and GFP-low using pMON30049 as a template, and then the resulting amplicons were diluted 1/50 and reamplified using primers GFP-up2 and GFP-low. The reactions were performed at 94°C 30 s, 58°C 30 s and 68°C 1 min programmed for 30 cycles and 35 cycles, respectively.

pGFP has two base mismatches with the single-stranded oligonucleotide SDO^{GFP} at the 35th and 38th position. They are both silent mutations and do not affect the expression of functional GFP, but will affect the pairing between the oligonucleotide and the targeted region of GFP construct. Using a QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instruction these mismatches were corrected to match the bases as in the oligonucleotide SDO^{GFP}. To make nonfunctional GFP mutants, a G to T change in the sixth codon was introduced to form a TAG stop codon, or a deletion of G at the same position to form a frame-shift mutant. These mutants were created using the site-directed mutagenesis method

a Correction oligonucleotide:

SDO^{GFP} 1 11 21 31 41
 5'-CCACC ATG GTG AGC AAG GGC GAG GAG CTG TTC ACC GGG GTG-3'

b Reporter vector:

pGFP ... CCACC ATG GTG AGC AAG GGC GAG GAG CTG TTC ACT GGC GTG ...

pGFPΔ ... CCACC ATG GTG AGC AAG GGC ΔAG GAG CTG TTC ACC GGG GTG ...

pGFPΔM2 ... CCACC ATG GTG AGC AAG GGC ΔAG GAG CTG TTC ACT GGC GTG ...

pGFPX ... CCACC ATG GTG AGC AAG GGC TAG GAG CTG TTC ACC GGG GTG ...

pGFPXM2 ... CCACC ATG GTG AGC AAG GGC TAG GAG CTG TTC ACT GGC GTG ...

Fig. 1 Reporter constructs and the correction oligonucleotide. **a** The sequence of the single-stranded DNA oligonucleotide with 5'-end labeled with Cy3 and 3'-end with inverted cytosine base. **b** Partial sequences of reporter GFP vectors showing the start of GFP coding sequence; ATG (dot underlined) is the initial codon of the GFP gene. pGFP contains a functional GFP sequence. pGFPΔ and pGFPΔM2

are deletion mutants with the deletion indicated as a Δ symbol and double underline. pGFPX and pGFPXM2 are the point mutation with a T (double underlined) substituting for G to create a stop codon. Both pGFPΔM2 and pGFPXM2 have two additional mismatches (single underlined) with the SDO^{GFP}. All GFP coding sequence was written in codon format

described above. All resulting constructs were sequenced to confirm that they contained only the desired mutations. The names and mutations of these constructs were shown in Fig. 1b.

Plant material and culture medium

Wheat (*Triticum aestivum*) varieties Bobwhite 26 (CIM-MYT) and Chinese Spring were used. The plants were grown in the greenhouse with 22°C/14°C day/night temperature and 16-h photoperiod. Caryopses 14–16 days after anthesis were surface-sterilized with 70% ethanol for 5 min followed by 50% of commercial grade disinfectant Zixo (Campbell Consumer Products, Australia) for 10 min, followed by rinsing four times in sterilized water. Immature embryos were aseptically removed and placed on the culture medium with the scutellum side up.

The basal culture medium used in this study was MSR, containing MS salts and vitamins (Murashige and Skoog 1962), 40 mg/l thiamine, 150 mg/l L-asparagine, 3% sucrose and 0.3% gelrite (Sigma) at pH 5.8. Medium MSC was MSR with 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) added. MSCO was an osmotic medium which had the same ingredients as MSC except that 240 g/l (0.7 M) of sucrose was added.

DNA delivery

The wild-type GFP construct pGFP and mutated GFP constructs were delivered either alone or with targeting oligonucleotide to the scutellum cells of freshly dissected immature embryos of wheat. A total of 5 μl DNA (2 μg plasmid DNA alone or 2 μg plasmid DNA and 2 μg oligonucleotide) was co-precipitated with 50 μl 2.5 M CaCl₂ and 20 μl 0.1 M spermidine onto 50 μl (1 mg)

0.6 μm gold particles (Bio-Rad) that were then washed once in 100% ethanol and then resuspended in 50 μl 100% ethanol. An aliquot (5 μl) of the resuspended gold particles was bombarded using a Bio-Rad Biolistic® PDS-1000/He delivery system to the freshly isolated wheat immature embryos arranged on the center of 6 cm Petri dish in a 1 cm circle. The bombardment was performed at 1100 psi, 27 mmHg vacuum and with the embryos at a 6 cm target distance.

Microscopy

For green fluorescent cell imaging, an inverted fluorescence microscope (Leica DMIL) was used. Filter set I3, which contains a 450–490 nm excitation filter, a 510 nm dichromatic mirror and a 515 nm LP barrier filter, was used for GFP imaging. Filter set N2.1, which contains a 515–560 nm excitation filter, a 580 nm dichromatic mirror and a 590 nm LP barrier filter, was used for imaging cells receiving Cy3 labeled oligonucleotide coupled to gold particles. A confocal microscope (Radiance 2000, Bio-Rad) and Lasersharp 2000 software were used for verifying the number of fluorescent cells in a scutellum one day after bombardment of pGFP. The number of restored green fluorescent cells on scutella was obtained 2 days after co-bombardment with mutant GFP construct and SDO^{GFP} and based on the counting of 100 scutella on MSC and MSR medium and 50 scutella on MSCO medium.

Results

GFP expression and observation of gene repair events

To verify that the reporter plasmid DNA and oligonucleotide SDO^{GFP} were delivered to the wheat scutellum

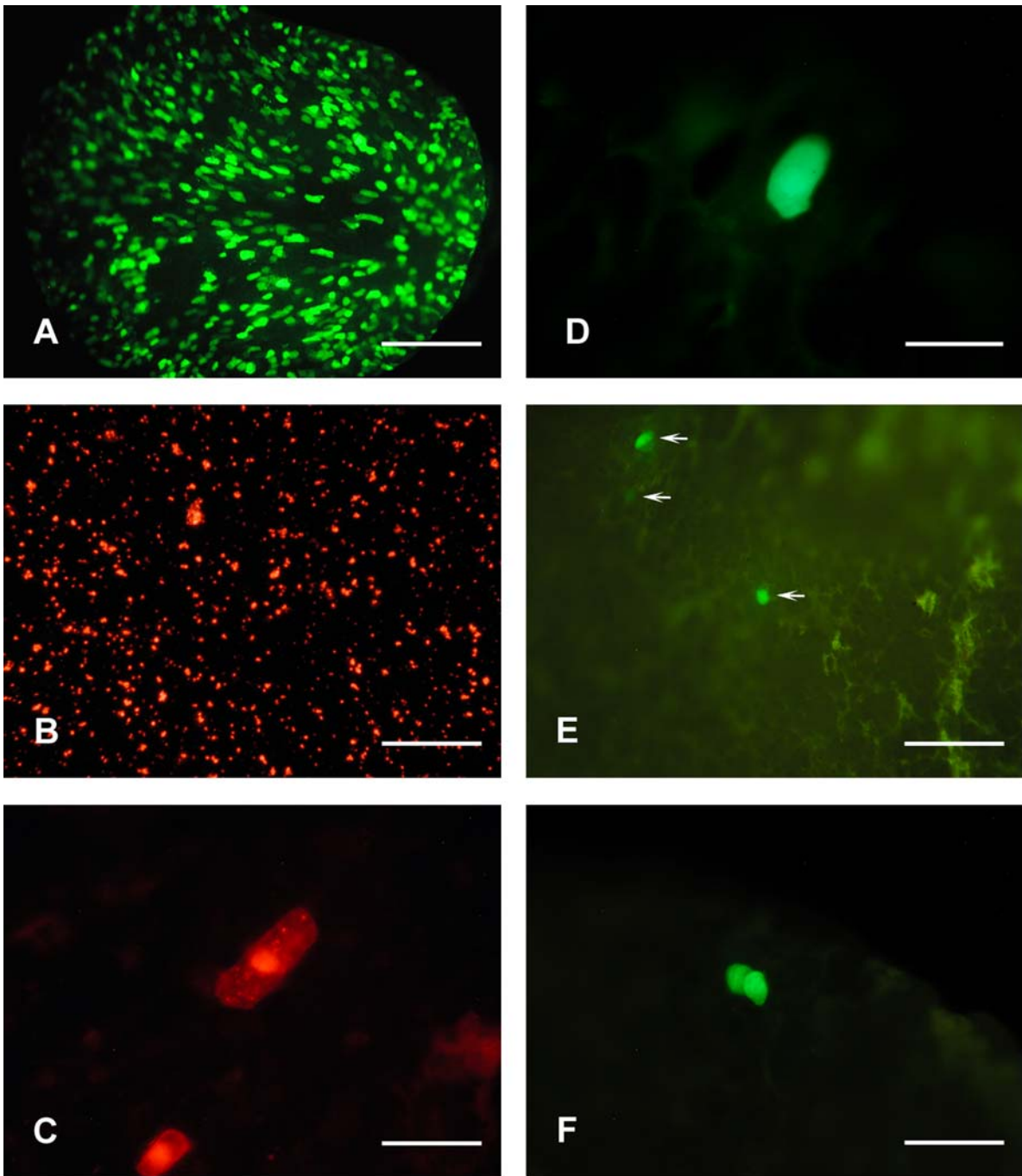


Fig. 2 **A** Scutellum side of an immature wheat embryo bombarded with control plasmid pGFP showing cells expressing GFP. **B** Fluorescence of gold particles after binding of Cy3-labeled oligonucleotide and dried onto a microscope slide. **C** Scutellum cells with Cy3 fluorescence immediately after bombardment with the gold particles. **D**,

E, **F** GFP expressing cell(s) after co-bombardment with mutant plasmid pGFP Δ and the oligonucleotide SDO^{GFP} into different scutellum **D**, **E** And **F**. Scale bar is 180 μ m in **A**, 72 μ m in **E**, 36 μ m in **F** and 18 μ m in **B**, **C** and **D**

cells, the plasmid pGFP alone, the SDO^{GFP} alone, or the combination of pGFP and SDO^{GFP} were coupled to gold particles and then bombarded into the scutellum cells of freshly dissected immature embryos cultured on MSC medium. Transient expression of GFP in the scutellum cells was observed with a fluorescence microscope 18 h after bombardment. There was no significant difference between the scutella bombarded with pGFP alone and the

scutella bombarded with pGFP plus SDO^{GFP} when the average number of fluorescent cells was compared (Fig. 2A), indicating that co-bombardment of plasmid pGFP and the oligonucleotide SDO^{GFP} did not affect the delivery of pGFP or the expression of GFP. The efficiency of coupling Cy3 labeled SDO^{GFP} to the gold particles was assessed by the observation of red fluorescence associated with excitation of the Cy3 fluorochrome using the fluorescence micro-

scope. One microliter of the resuspended gold particles was dropped onto a glass slide and observed with the fluorescent microscope; almost all of the gold particles had red fluorescence (Fig. 2B). This pattern was not altered by addition of the plasmid pGFP. Cells that received Cy3 labeled SDO^{GFP} were also clearly observed after bombardment with SDO^{GFP} alone or with pGFP and SDO^{GFP} together (Fig. 2C).

Initial observation of gene repair events was achieved by using pGFPΔM2 and RDO^{GFP} or SDO^{GFP}. The plasmid DNA pGFPΔM2 with either RDO^{GFP} or SDO^{GFP} was bombarded into freshly isolated immature wheat embryos on MSC medium. On the following three days, gene repair events were observed by the restoration of green fluorescent cells. In the case of RDO-directed gene repair, one green cell was observed among 100 scutella on the first day, and two green cells were observed on the second day. In the case of SDO-directed gene repair, 14 green cells were observed among 100 scutella on the first day, and 33 green cells were observed on the second day. On the third day, the number of green cells decreased, and observation became difficult because of the cell proliferation. In the control (pGFPΔM2 alone), no green cells were observed during the first 3 days after bombardment. Figure 2D–F shows cells expressing functional GFP after receiving pGFPΔ and SDO^{GFP} in different scutella. The restored fluorescence is weaker than the cells transformed with wild-type pGFP. It can be expected that a bombarded cell contains multiple copies of a gene construct, but gene repair occurs only in a few copies. The brightness of restored green fluorescence varied between cells where gene repair had occurred (Fig. 2E). This appeared to be due to the location of the cell in the epidermal or in the subepidermal layers. Confocal microscopy of the scutellum after receiving the wild-type pGFP showed that the first three layers of cells received the plasmid DNA after bombardment (data not shown). The number of copies of the plasmid DNA in a cell, which had been converted to and expressed a functional GFP, may also contribute to the brightness of green fluorescence, although this could not be determined in the current assay. Figure 2F shows two adjacent cells had restored functional GFP. It is most likely that cell division occurred after one cell had gene repair. Taken together, these results demonstrated that both RDO and SDO were capable of functional correction of the plasmid gene in living wheat cells and confirmed the feasibility of the transient reporter assay system.

Efficiency of gene repair on point mutation and deletion mutation

On the basis of the initial assessment, the SDO^{GFP} was more capable of plasmid gene correction in the assay system than the RDO^{GFP}, and was therefore used in the following experiments. The next series of experiments were all observed on the second day (40–48 h) after co-bombardment with the different mutant GFP constructs and SDO^{GFP} and scored for GFP restoration. For each treatment, 100 immature embryos were included to minimize experimental

error. In experiment A (Table 1), the frequency of correction of the point mutation pGFPX was almost twice that of the deletion mutation pGFPΔ. The plasmids pGFPXM2 and pGFPΔM2 which had two additional mismatches with the correction oligonucleotide SDO^{GFP} were also included in the experiment. Interestingly both of them showed much lower repair efficiency than that of pGFPX and pGFPΔ, but no significant difference between the deletion mutant pGFPΔM2 and the point mutant pGFPXM2. It is likely that the extra mismatches reduced the pairing of SDO^{GFP} to the target DNA, which potentially became the rate-limiting step for repair. The experiments were repeated in B and E (Table 1). The experiment B showed similar gene repair efficiencies as in experiment A in all four different treatments. However, the repair efficiencies were much lower in experiment E compared to experiments A and B. It is possible that the scutella material used in experiment E was not as competent for gene repair as the scutella in A and B. Although the overall repair frequency was lower in experiment E, the repair efficiency of the point mutation (pGFPX) was still about two times higher than that of the deletion mutation (pGFPΔ).

This large variation between individual experiments made the standard deviation very high in Table 1. It is possibly due to the difference in biological activity of the immature embryos used in different experiments (see Discussion section). It is important to compare the effect of different treatments on the gene repair efficiency within the same experiment.

Culture medium affects the gene repair efficiency

In experiment B, three different media were used to culture the immature embryos. The high osmotic medium MSCO strikingly increased the repair efficiency of the correction of the point mutation, while MSR medium which had no plant growth regulator added, clearly had the lowest repair efficiency (Table 1). The experiments were repeated in C, D, E, F and G. Although there was variation between the experiments, the osmotic medium did increase the overall gene repair frequency in wheat scutellum cells. An interesting phenomenon was that some embryos have very high gene repair ability. In experiment A, among 100 embryos, one showed 24 green fluorescent cells, while other embryos showed 0, 1, 2, 3, 4 or 5 green cells after co-bombardment with pGFPX and SDO^{GFP}. In experiment B, one embryo showed 130 green cells and two showed 40 and 38 green cells, while others only had 1–10 green cells on MSCO medium after co-bombardment with pGFPX and SDO^{GFP}. In most experiments, there were always one or two embryos showing 5–10 times more green fluorescent cells than the rests. This suggests that some embryos were more competent for oligonucleotide-directed gene repair than others at the time of oligonucleotide delivery.

When comparing the effects of different culture media on the gene repair efficiency, we need to account for the potential increase of DNA uptake and GFP expression. To confirm the influence of different media, wild-type pGFP

Table 1 The number of fluorescent cells observed in 100 scutella after bombardment with mutant GFP constructs and the correction oligonucleotide

	MSC						MSR						MSCO ^a					
	Experiment			Experiment			Experiment			Experiment			Experiment			Experiment		
	A	B	E	Mean ± SD	B	E	F	Mean ± SD	B	C	D	E	F	G	Mean ± SD			
pGFPΔM2+SDO ^{GFP}	29	32	4	22±15	5	1	0	2±3										
pGFPΔ+SDO ^{GFP}	68	61	18	49±27	11	7	0	6±6										
pGFPXM2+SDO ^{GFP}	33	28	7	23±14					318	180	116	540	246	234	340±173			
pGFPX+SDO ^{GFP}	128	88	32	83±48					1212	572	560	530	462	648	664±275			

SD is the standard deviation between individual experiments

^aIn MSCO, 50 scutella were counted, and the numbers doubled, because of very high numbers observed

Table 2 The average number and standard deviation of green fluorescent cells per scutellum

	Medium		
	MSC	MSR	MSCO
Green cells (wild-type GFP)/scutellum	326±162	103±57	1068±488
Frequency of repair of pGFPX (%)	0.25	ND	0.62
Frequency of repair of pGFPΔ (%)	0.15	0.06	0.32

The data were observed on 10 medium size scutellum (1–1.3 mm) after bombardment with the wild-type GFP construct, and frequencies of gene repair, calculated as the average number of cells that had gene repair (Table 1) among the average number of cells receiving and expressing wild-type GFP on each medium

was bombarded into the scutella of immature wheat embryos using the same amount of plasmid DNA as in the gene repair experiments. Cells expressing GFP were observed and counted on the following day of the bombardment. The average number of cells expressing GFP was shown in Table 2. It was obvious that different media also affected the expression of the wild-type GFP. There were about 10 times and 3 times more green cells on osmotic medium than that on MSR and MSC medium, respectively. Given these uptake and expression effects, the frequencies of targeted gene repair were calculated to show the number of cells capable of oligonucleotide-directed gene repair after receiving and expressing the reporter plasmid DNA and the targeting molecule SDO^{GFP}. Table 2 shows that the repair frequency of point mutation was 0.25% on MSC and 0.62% on MSCO, 2.5 times higher on MSCO than on MSC. The frequency of repair of the deletion mutation was 0.32% on MSCO and 0.15% on MSC; two times higher on MSCO medium than on MSC. Overall the frequency of repair of the point mutation was nearly two times higher than that of repair of the deletion mutation. The absolute frequency of gene repair on the DNA level cannot be determined because the total number of copies of plasmid DNA in scutellum cells and the number of copies of the plasmid converted to functional GFP plasmid was not determined in this assay.

The wheat line “Bobwhite 26” was used in all experiments. However, in experiments B and C, the variety Chinese Spring was also included. In experiment B, immature embryos of Chinese Spring were cultured on MSC medium and were co-bombarded with pGFPX and SDO^{GFP}, none of the embryos showed any green fluorescent cells. In experiment C, Chinese Spring was repeated with culturing on MSCO medium. Twenty-one green cells were observed among 100 embryos. This was very low compared to 572 green cells observed in Bobwhite 26 of the same experiment (Table 1). These results indicated that different varieties of wheat may also have different gene repair capabilities.

Discussion

Oligonucleotide-directed gene repair is considered to be either an alternative or a better technology for rapid trait development or reverse genetics than traditional gene transformation or gene targeting via homologous recombination (Puchta 2002; Britt and May 2003). Oligonucleotide-directed DNA conversion of point, insertion or deletion mutations on the targeted chromosomal position overcomes the problems associated with transgene instability and variable expression levels due to the random nature of transgene integration. Homologous recombination, especially in higher plants, is characterized by low frequency and limited success (see review Hanin and Paszkowski 2003). The early success of oligonucleotide-directed gene conversion in tobacco (Beetham et al. 1999) and maize (Zhu et al. 1999, 2000) demonstrated that altered genes can be stably maintained through mitosis and transmitted through meiosis in a Mendelian fashion to subsequent generations. The frequency of targeted gene repair in maize was approximately 1 in 10^4 (Zhu et al. 1999). However, the later experiments in tobacco cells showed a lower frequency of 1 in 10^6 (Kochevenko and Willmitzer 2003). In mammalian cells, there is a large variation in the repair frequency reported by different laboratories (Cole-Strauss et al. 1996; Kren et al. 1997; van der Steege et al. 2001), and even in the same laboratory using the same batch of oligonucleotide and cell line (Alexeev and Yoon 1998). Although the quality and quantity of oligonucleotides delivered to cells are among important factors for achieving a successful gene repair, the large variation in reported repair frequency indicates that many other factors may be involved. Different cell types and different physiological stages of cells may have different repair activity. The biological activities of both DNA pairing and repair in a target cell and the status of DNA replication and transcription of the targeted gene are known to influence the gene repair process (Drury and Kmiec 2003; Igoucheva et al. 2002, 2003; Brachman and Kmiec, 2004). It is therefore important to test the repair ability of the target cells before extensive gene-targeting experiments are undertaken.

In the present study, a mutated nonfunctional plasmid GFP gene can be corrected to restore GFP function by introduction of single-stranded DNA oligonucleotides into cultured wheat scutellum cells. The restored functional GFP can easily be detected by the green fluorescence in the cell. This method can be readily and quickly employed to assay gene repair activity in cells of a given cell type grown in specific culture conditions. This is a useful assay system to test the feasibility of oligonucleotide-directed gene alteration.

Our results showed that 2,4-D and an osmotic treatment dramatically increased the gene repair frequency in wheat. The growth regulator 2,4-D is a synthetic auxin commonly used in tissue culture to induce somatic embryogenesis in cereal transformation. It plays a role in the activation of genes involved in cell de-differentiation and division (Dudits et al. 1991; Wernicke and Milkovits 1987). Induc-

tion of cell division and somatic embryogenesis includes activation of many biological activities, such as chromosomal remodeling, changes of transcriptional and translational profiles and other biochemical and physiological changes (for review, see Fehér et al. 2003). This may also include changes with DNA pairing and DNA repair activity as suggested in this study. Auxin treated scutellum has increased cell division activity, which means more cells are at DNA replication stage than untreated scutellum cells. DNA replication will alter or disrupt the chromatin structure, which could make the targeted DNA region more accessible to oligonucleotide pairing and gene repair and then increase the targeting frequency as found in mammalian cells (Brachman and Kmiec 2004). Osmotic treatment during the Biolistic[®] bombardment of plant cells is used to reduce tissue damage (Kemper et al. 1996). In this study, the number of green cells observed on MSC, MSR and MSCO medium after bombardment of wild-type pGFP was different. These differences are most likely due to the biological activity of the cell and the level of damage to the cell. With no hormonal and osmotic treatment (MSR), cells are expected to have the least biological activity and the most damage, so it was showed the lowest number of cells expressing GFP after the bombardment. With 2,4-D treatment (MSC) the number of green cells increased. With both 2,4-D and osmotic treatments (MSCO), it was observed the highest level of expressing GFP cells and highest frequency of oligonucleotide-directed gene repair.

Variation was observed between experiments and among individual embryos in the same experiment, reflecting that the biological and physiological states of the immature embryos may not always be the same. In fact, regeneration ability of immature wheat embryos was found to be different from batch to batch even though they were grown under the same greenhouse conditions and cultured in the same way (data not shown). Oligonucleotide-directed gene repair is a complex biological process needing the oligo to reach the targeted DNA in a cell which has DNA pairing and DNA repair activity. All these processes are not easily controlled. Cell division in a cultured scutellum is not synchronized, so cells are at different stages of a cell cycle. It is possible that different stages of a cell cycle may have different DNA pairing and DNA repair activity which influences oligonucleotide-directed gene repair. The variation of gene repair frequency found in this study may reflect variation between scutella for active cell division. It also reflects the complexity of this repair process consistent with the observation of repair frequency of 0.01–15% in correcting a point mutation in mouse tyrosinase gene among at least 30 independent experiments (Alexeev and Yoon 1998; Igoucheva et al. 2004).

The repair efficiency of a point and a frameshift (deletion) mutation was compared in this study. Repair of the point mutation was twice as efficient as that of the deletion mutation in wheat cells. This was similar to oligonucleotide-targeted repair studies in yeast (Liu et al. 2001, 2002a). In yeast, repair of an episomal point mutation was most efficient and was approximately twice that of repair of an insertion mutation, and fivefold better

than repair of a deletion mutation. Variable efficiencies of repair of a point, insertion or deletion mutation were also reported in an *in vitro* assay system using plant “cell-free” extracts (Rice et al. 2000; Kmiec et al. 2001). However, these data cannot be directly compared with the data presented here, as our study is the first report of *in vivo* correction directly reporting differences between substitution and deletion DNA repair. The frequency of gene repair reported in this study is the frequency of cells capable of oligonucleotide-directed gene repair of a point or deletion mutation in the first 48 h after receiving the reporter plasmids and targeting oligonucleotides.

Using a plasmid gene repair assay system, it is possible to detect directly phenotypic changes upon gene correction. This is widely used in bacterial, yeast and mammalian cell cultures (Igoucheva et al. 2004). In plants, this is the first report using a plasmid gene repair assay system. The results observed here represent plasmid DNA repair activity of *in vitro* cultured wheat scutellum cells. The plasmid DNA when introduced into a mammalian cell is not integrated into the genome and exists as extra-chromosomal DNA for a period of time. This type of DNA in a mammalian cell may have very different structure from chromosomal DNA, and is generally more open and accessible than cellular chromatin (Smith and Hager 1997). It may also be true in plant cells. The mechanisms involved in episomal or plasmid gene correction after transient transfection of high eukaryotic cells may differ from genomic correction. However, the episomal/plasmid repair assay system is still a valuable system, and normally shows 10- to 100-fold higher efficiency than chromosomal repair (Liu et al. 2001; Igoucheva et al. 2001; Pierce et al. 2003; Nickerson and Colledge 2003). Therefore, the results observed in this study can provide useful information for genomic targeting strategies.

The method presented in this study could be extended to study chromosomal gene correction after producing a stable transgenic wheat plant harboring a mutated GFP gene inserted in the genome. Targeted alteration of genomic DNA in mammalian cells by RDO or SDO occurs at a low frequency (\sim or $<10^{-4}$) (Alexeev et al. 2002) and even lower in plant cells (Kochevenko and Willmitzer 2003). A more sensitive assay system must be used in the study of chromosomal correction. If the GFP recovery system is to be used in the assay of chromosomal DNA repair, a suspension cell culture or protoplast culture would be appropriate as a targeting system, which will allow the detection of restored green fluorescence to be analyzed by FACS (fluorescence activated cell sorting), which could detect infrequent gene repair events more sensitively and efficiently (Thorpe et al. 2002).

In summary, this is the first report of oligonucleotide-directed gene conversion of a plasmid gene in wheat scutellum cells, and the first report of using a plasmid gene repair assay to study the gene repair activity in living plant cells. The efficiencies of repair of a point mutation and a deletion mutation, as well as the effects of different culture media were compared. This assay system can be extended to measure the capacity of different plant types or tissues to catalyze gene repair events. This study provided useful

information on selection of plant materials, culture conditions and physiological stages of cells suitable for genetic alteration using oligonucleotide-directed mutagenesis.

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