Regeneration of Haploid Plants After Distant Pollination of Wheat Via Zygote Rescue

Ferenc Bakos, Katalin Jäger, and Beáta Barnabás[*]

*Agricultural Research Institute of the Hungarian Academy of Sciences, P.O.B. 19,
H–2462, Martonvásár, Hungary*

Haploid wheat plants have been produced by a new method of zygote rescue carried out after distant pollination. Wheat stigmas were pollinated with maize pollen and subsequently the activated egg cells from the elongated ovaries were rescued for in vitro plant development in single cell culture. As the control, 2-week-old embryos were also dissected and then cultured. The efficiency of both techniques was comparable. Wheat was also pollinated with rice, and the further development of rescued zygotes into multicellular structures is reported here for the first time. Because the lack of a normal endosperm hampers embryo development even in the early stages, early zygote rescue (two days after distant pollination) may represent a more efficient way of producing double haploid (DH) plants in cultivars that are recalcitrant in androgenic cultures, after further optimization of in vitro culture of isolated single cells.

Key words: Activated egg cell, co-culture, double haploid, embryo rescue, haploid, nurse culture, parthenogenesis, wheat × maize, zygote rescue.

INTRODUCTION

The production of (doubled) haploid plants is advantageous for plant breeders because it offers a way of rapidly advancing chosen lines to complete homozygosity, and of increasing the efficiency of subsequent selection (Kisana et al., 1993; Sharma et al., 2002). Although various methods (delayed pollination, pollination with unviable pollen grains, chemical induction of parthenogenesis) have been attempted in cases where micropore embryogenesis cannot be applied efficiently (e.g., recalcitrant *T. durum*, *T. triticale* and bread wheat genotypes), pollination with a distantly related species appears to present a real alternative technique for generating haploids by inducing embryogenic development (O'Donoughue and Bennett, 1994; Wedzony et al., 1998; Laurie and Bennett, 1988). The method of distant pollination has been successfully applied also when wheat lines containing one unpaired barley chromosome needed to be advanced into stable additional lines, which contain two homologous chromosomes of the barley (Linc and Molnár-Láng, 2003). In most cases of distant pollination, certain genotypes of maize have been used as interspecific pollinator (Verma et al., 1999; Singh et al., 2004). In egg cells fertilized as a result of distant pollination, the chromosomes of the distantly related sperm cell start to be eliminated during the first divisions, leading to the induction of embryogenic development (Laurie and Bennett, 1989, 1990; Mochida et al., 2004). These studies show that egg cell activation and subsequent embryo development in wheat can result from fertilization by the sperm cell of not only its own species but a distantly related species as well. Central cell fertilization also occurs, but the simultaneous development of the embryo and endosperm, suggesting double fertilization, is very rare (Laurie and Bennett, 1989; Zhang et al., 1996). Even if both cells are fertilized, the imbalance between the maternal and paternal genomes impedes endosperm development (Lin, 1984; Haig and Westoby, 1991), and in consequence the growth of the young embryo is inhibited (Zhang et al., 1996). Zygotic embryo and endosperm development in wheat crossed with a distantly related species (maize) has been cytologically investigated (Wedzony and van Lammeren, 1996; Zhang et al., 1996), but the biochemical interactions between the alien sperm cell and the egg cell, and between the endosperm and the embryo, have not yet been clarified. Similarly, little data is available on the genes and epigenetic factors responsible for the embryonic development of activated egg cells lacking the paternal genome from the corresponding species. Only

---

*e-mail: bea@mail.mgki.hu

TABLE 1. In planta and in vitro development of zygotes obtained after pollinating wheat with maize. The ratio of pistils/zygotes reaching the stated developmental stages is shown for each cross; (n = 3). Data are shown as percentages of pollinated florets *Data of the obtained plants followed by the same letter do not significantly differ at p = 0.05

Wheat × maize crosses	Pollinated florets	Frequency of elongated pistils %	In planta embryos %	In vitro cell division %	In vitro embryo formation%	*Regenerated plants %
Embryo rescue						
Mv Ködmön × Norma SC	308 ± 30	62.3 ± 2.8	27.6 ± 1.4			7.5 ± 1.3[a]
Mv Pálma × Norma SC	297 ± 17	27.9 ± 1.4	9.2 ± 1.1			0.7 ± 0.3[b]
Zygote rescue						1.6 ± 0.9[b]
Mv Ködmön × Norma SC	142 ± 16	57.5 ± 4.2		20.9 ± 6.6	1.9 ± 0.7	
Mv Pálma × Norma SC	139 ± 18	26.1 ± 4.9		16.5 ± 2.9	4.1 ± 1.6	3.6 ± 2.0[ab]

one team has reported research on the gene expression pattern of haploid parthenogenic egg cells and developing embryos (Kumlehn et al., 2001), but these were not originated from distant pollination. The majority of publications report molecular biological studies on endosperm formation and on autonomous embryo development from the unreduced embryo sac (for review: Koltunow and Grossniklaus, 2003).

In the absence of a normal endosperm, embryo rescue is needed, because embryos not excised within 3 weeks will perish (Laurie and Bennett, 1988; Zhang et al., 1996). In a number of studies, embryos have been rescued at a late stage, 2–3 weeks after distant pollination (Laurie and Bennett, 1988; Singh et al., 2001; Sharma et al., 2002). In the present study, early rescue was applied in an attempt to culture zygotes or proembryos that had not been exposed to the negative influence of endosperm abnormality or absence for a long period. Dissected, activated egg cells were cultured with young wheat pistils as nurse tissue (Bakos et al., 2003). Embryo rescue and early zygote rescue, followed by nurse culturing, were also attempted in wheat × rice crosses, from which haploid wheat plants have never yet been obtained.

MATERIALS AND METHODS

Wheat and maize plants were grown in phytotron chambers as described by Tischner et al. (1999). Rice seeds were germinated at 26˚C for one week, then transferred to soil. The temperature during the 3-month climate program gradually increased by 1˚C/week from 20/18˚C to 28/26˚C day/night. Light intensity was 400 μmol m^{-2} s^{-1}, with a 12 h photoperiod.

For induction of egg cell activation, emasculated wheat spikes (see Tables 1 and 2 for cultivars) were hand-pollinated with maize (Mv Norma SC) and rice (see Table 2 for genotypes). One day after pollination, each pistil was treated with one drop of 3 mg/l 2,4-D solution containing 100 mg/l AgNO$_3$ (O'Donoughue and Bennett, 1994).

For embryo rescue and nursing, activated egg cells were isolated 2 days after pollination (DAP) as described by Kovács et al. (1994) and then co-cultivated in the presence of wheat ovaries as nurse cells according to Bakos et al. (2003). As the control, embryos were dissected 14 DAP and grown on agar-solidified B5 medium without nurse cells. Three independent experiments were performed in the case of wheat × maize pollination, and one in the case of wheat × rice crosses. Photographs were taken with an Olympus inverted microscope using an Olympus Camedia C3030 Zoom digital camera.

The ploidy level was determined by flow cytometric analysis according to the protocol elaborated by Galbraith et al. (1983) and modified by Szakács and Barnabás (2004).

RESULTS

Zygotes rescued after maize pollination did not divide in planta prior to dissection (Fig. 1a). During the first few divisions, multicellular structures developed. Usually these structures contained cells with optically dense cytoplasm, and their cells were arranged in lobes (Fig. 1b). Except for this initial stage, the development of the zygotes resembled the regular in vivo embryonic pathway (Fig. 1c,d). However, twin or multiple embryos were frequently formed, perhaps as a consequence of cleavage during the initially irregular cell divisions (Fig. 1b,c). As a result of in vitro development, green plants with sterile spikes were obtained (Fig. 1e-g).

TABLE 2. In planta and in vitro development of zygotes obtained after pollinating wheat with rice. The number of pollinated flowers and the ratio of pistils/zygotes reaching the stated developmental stages are shown for each wide cross (n = 1) *In planta embryoids: two-week-old embryos in size 0.3–0.8 mm

Wheat × rice crosses	Embryo rescue			Zygote rescue		
	Pollinated florets	Frequency of elongated pistils %	*In planta embryoids %	No. of pollinated florets	Frequency of elongated pistils %	In vitro cell division %
Chinese Spring × Rigola	558	69.9	1.6	171	65.5	1.8
Chinese Spring × Bioryza	326	66.6	1.5	212	73.6	1.4
Chinese Spring × Janka	138	59.4	0.0	132	58.3	0.0
Orofen × Rigola	82	15.9	0.0	224	13.4	0.0
Orofen × Bioryza	209	19.1	0.0	142	15.5	3.5
Orofen × Janka	170	35.3	0.0	232	17.7	0.0
Mv Pálma × Rigola	156	18.6	0.0	186	16.7	0.5
Mv Pálma × Bioryza	163	20.9	1.8	77	19.5	0.0
Mv Pálma × Janka	145	22.1	2.8	198	19.2	3.0

Green plants were also obtained in both wheat genotypes after embryo rescue and subsequent culture, although in this case the ratio of germinated embryos was much lower than for embryos developed in vitro (Tab. 1). The two-week-old embryos dissected from the ovaries varied greatly in size, and only the well-developed ones at least 1 mm in size were able to regenerate into plants, as also observed by Zhang et al. (1996). By means of early zygote rescue, 3.6 and 1.6 plants per 100 florets (1.0 and 0.4 plants per spike) were obtained in Mv Pálma and Mv Ködmön, respectively. Embryo rescue resulted in 0.7 and 7.5 plants per 100 florets (0.2 and 2.6 plants per spike) from the same genotypes, respectively (Tab. 1). Flow cytometric analysis showed all the plants to be haploid (data not shown).

In the case of wheat × rice pollinations, in planta embryo development was absent or very limited (Tab. 2). These embryoids varied in size, but most of them were very small and none of them were able to develop further on the culture medium (data not shown). However, when the activated egg cells were dissected two days after pollination, a few of them started to divide and grow when co-cultured with wheat ovaries (Fig. 2). They reached a multicellular stage but were unable to develop further (Fig. 2c,d).

DISCUSSION

The in planta development of activated egg cells obtained by wheat × maize pollination (Wedzony and van Lammeren, 1996) was already much slower in the very early stages than that of the zygotes, which start to divide within 24 hours after pollination with the same species (Batygina and Vasilyeva, 2001). This finding, and the relatively low survival of rescued zygotes and their reduced capacity for embryonic development, show that they have special, as yet unknown requirements in their early developmental stages, and that highly sophisticated conditions are needed to culture them efficiently. However, nearly all the isolated zygotes that started to complete the normal embryogenic pathway in vitro were able to grow into plants (Kumlehn et al., 1999; Bakos et al., 2003), even though the rescued zygotes frequently formed multiple embryos, as also observed by Zhang et al. (1996) and Kumlehn et al., (1999). Thus, early zygote rescue might be an alternative method for obtaining haploids via distant crosses. Since the lack of a functional endosperm hinders the development of embryos even at a very early stage (Wedzony and van Lammeren, 1996), the rescue of two-day-old zygotes may be advantageous, providing an efficient, well-optimized single-cell culture method. Although the efficiency of this method is already comparable to that of embryo rescue (e.g., Zhang et al., 1996; Sharma et al., 2002), further optimization of in vitro culture following early zygote rescue could improve the efficiency of this new method even further, since the embryos are rescued earlier from the severely suboptimal environment, allowing their faster development. This expectation is strengthened by the observation that the embryos obtained by rice pollination were very small even after 2 weeks and did not show any further development in culture, while the dissected, activated egg cells were able to perform several division cycles. Zygotes of rice have also been isolated (Han et al., 1998) and cultured (Zhao et al., 2000), but the most developed cells reached only the stage of multicellular
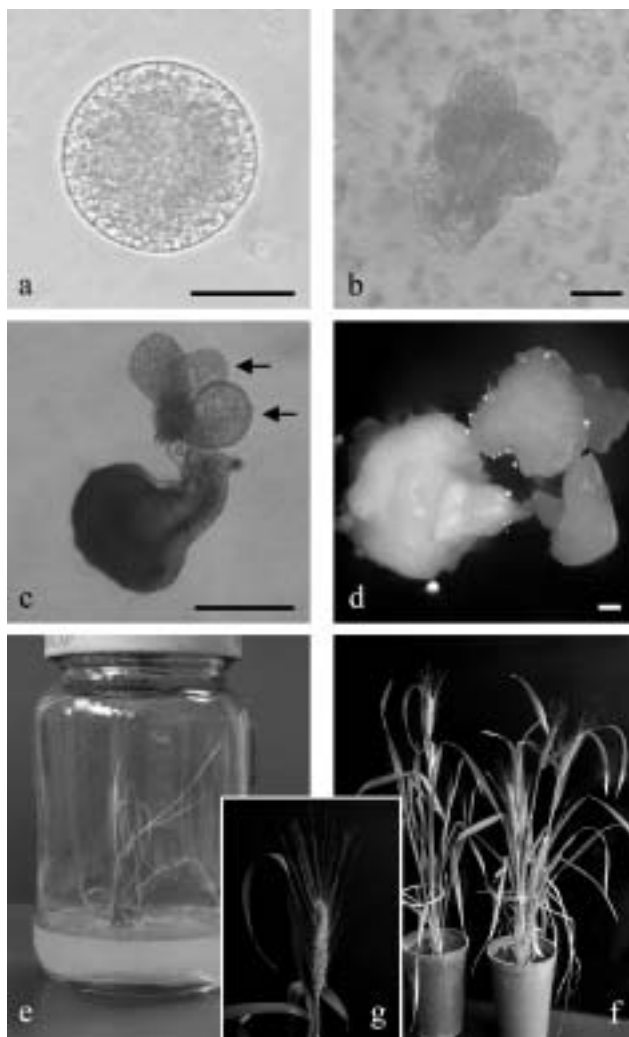
Fig. 1. In vitro development of activated egg cells of wheat rescued two days after pollination with maize. (**a**) Freshly rescued zygote, (**b**) Multicellular structure after the initial divisions of an activated egg cell 5 DAP, (**c**) Club-shaped multiple embryo 14 DAP. The individual embryos are attached to each other but are clearly distinguishable (arrows), (**d**) Dorsiventrally differentiated mature embryo 28 DAP, (**e**) Plantlet (Mv Pálma) on regeneration medium 44 DAP, (**f**) Adult haploid plant (Mv Ködmön) with sterile spikes (**g**). DAP – days after pollination. Bars = 25 μm in (a) and (b); 250 μm in (c) and (d).

Fig. 2. In vitro development of activated egg cells obtained by pollination with rice. (**a**) Freshly rescued zygote 2 DAP, (**b**) First in vitro cell division 4 DAP. Note the division plane (arrows), (**c**) Multicellular structure resulting from several division cycles 6 DAP, (**d**) A further developed multicellular structure 9 DAP. Note the cells that did not divide in culture (arrows). Possibly these egg cells were not activated by distant pollination. DAP – days after pollination. Bars = 25 μm.

structure, similarly to our results from wheat × rice crosses. Although earlier studies on the chemical composition of the wheat ovules revealed carbohydrates, amino acids and various ionic components (Mäes et al., 1999), further microanalyses of the microenvironment (ovule fluid) of zygotic embryos of cereals would greatly promote the efficiency of in vitro cultures for raising plants from rescued zygotes.

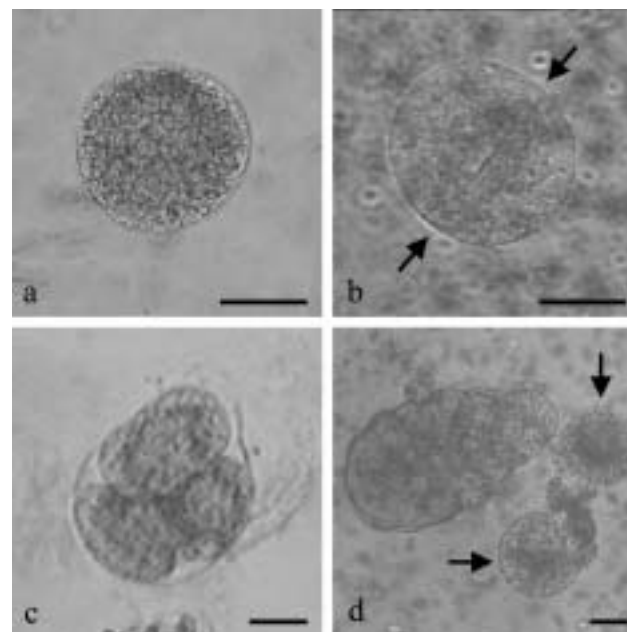In our experience, the incompatibility between wheat and rice is much stronger than between wheat and maize. To discover the mechanism of the existing incompatibility, further detailed cytological and molecular biological studies on wheat × maize (Wedzony and van Lammeren, 1996; Kumlehn et al., 2001) and especially on wheat × rice systems will be required. Since there is not yet any available information on the mechanism of egg cell activation of wheat following pollination with rice, the occurrence of the fertilization event needs to be confirmed by morphological studies of the embryo sac.

The results presented here provide a new starting point for improving the DH production efficiency of wheat genotypes that are recalcitrant in anther/isolated microspore culture. Since zygote culture is probably adaptable to durum wheat, this culture method, combined with maize pollination, could be helpful in durum wheat DH production, which is a serious problem for plant breeders at present.

REFERENCES

BAKOS F, DARKÓ É, PÓNYA Z, and BARNABÁS B. 2003. Application of wheat (*Triticum aestivum* L.) microspore culture and ovaries to raise wheat zygotes in vitro. *Acta Biologica Cracoviensis Series Botanica* 45: 107–110.

BATYGINA TB, and VASILYEVA VE. 2001. In vivo fertilization. In: Bhojwani SS and Soh WY [eds.], *Current trends in the embryology of Angiosperms*, 101–142. Kluwer Academic Publishers, Dordrecht, The Netherlands.

GALBRAITH DW, and SCHULTZ J. 1983. Rapid flow cytometric analysis of nuclear DNA levels in higher plants. In: Downey K, Voellmy RW, and Ahmad F [eds.], *Advances in gene technology: Molecular genetics of plants and animals,* 552. Academic Press, New York, USA.

HAIG D, and WESTOBY M. 1991. Genomic imprinting in endosperm – its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Philosophical Transactions of the Royal Society of London Series B – Biological Sciences* 333: 1–13.

HAN HM, ZHAO J, SHI HZ, YANG HY, and ZHOU C. 1998. Isolation of egg cells and zygotes in *Oryza sativa. Acta Botanica Sinica* 40: 186–188.

KISANA NS, NKONGOLO KK, QUICK JS, and JOHNSON DL. 1993. Production of doubled haploids by anther culture and wheat × maize method in wheat breeding program. *Plant Breeding* 110: 96–102.

KOLTUNOW AM, and GROSSNIKLAUS U. 2003. Apomixis: A developmental perspective. *Annual Review of Plant Biology* 54: 547–574.

KOVÁCS M, BARNABÁS B, and KRANZ E. 1994. The isolation of viable egg cells from wheat (*Triticum aestivum* L.). *Sexual Plant Reproduction* 7: 311–312.

KUMLEHN J, LÖRZ H, and KRANZ E. 1999. Monitoring individual development of isolated wheat zygotes: a novel approach to study early embryogenesis. *Protoplasma* 208: 156–162.

KUMLEHN J, KIRIK V, CZIHAL A, ALTSCHMIED L, MATZK F, LÖRZ H, and BÄUMLEIN H. 2001. Parthenogenetic egg cells of wheat: cellular and molecular studies. *Sexual Plant Reproduction* 14: 239–243.

LAURIE DA, and BENNETT MD. 1988. The production of haploid wheat plants from wheat × maize crosses. *Theoretical and Applied Genetics* 76: 393–397.

LAURIE DA, and BENNETT MD. 1989. The timing of chromosome elimination in hexaploid wheat × maize crosses. *Genome* 32: 953–961.

LAURIE DA, and BENNETT MD. 1990. Early post-pollination events in hexaploid wheat × maize crosses. *Sexual Plant Reproduction* 3: 70–76.

LIN BY. 1984. Ploidy barrier to endosperm development in maize. *Genetics* 107: 103–115.

LINC G, and MOLNÁR-LÁNG M. 2003. Two methods for the development of wheat/barley disomic additions in winter wheat varieties, and identification using molecular cytogenic techniques (C-banding, GISH). *Növénytermelés* 52: 1–13.

MÄES O, COMEAU A, NADEAU P, SIMARD RR, and ST-PIERRE CA. 1999. Characterization of the natural chemical and osmotic environment of early wheat embryogenesis. *Physiologia Plantarum* 107: 220–229.

MOCHIDA K, TSUJIMOTO H, and SASAKUMA T. 2004. Confocal analysis of chromosome behavior in wheat × maize zygotes. *Genome* 47: 224–228.

O'DONOUGHUE LS, and BENNETT MD. 1994. Durum-wheat haploid production using maize wide-crossing. *Theoretical and Applied Genetics* 89: 559–566.

SHARMA H, YANG Y, and OHM H. 2002. An assessment of doubled haploid production in soft red winter wheat by wheat × corn wide crosses. *Cereal Research Communications* 30: 269–275.

SINGH S, BEHL RK, and PUNIA MS. 2001. Production of double haploids via maize pollination in wheat. *Cereal Research Communications* 29: 289–296.

SINGH S, SETHI GS, and CHAUDHARY HK. 2004. Differential responsiveness of winter and spring wheat genotypes to maize-mediated production of haploids. *Cereal Research Communications* 32: 201–207.

SZAKÁCS É, and BARNABÁS B. 2004. Development of diploid pollen in spikelet cultures of barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.). *Acta Agronomica Hungarica* 52: 1–8.

TISCHNER T, KŐSZEGI B, and VEISZ O. 1997. Climatic programmes used in the Martonvsr phytotron most frequently in recent years. *Acta Agronomica Hungarica* 45: 85–104.

VERMA V, BAINS NS, MANGAT GS, NANDA GS, GOSAL SS, and SINGH K. 1999. Maize genotypes show striking differences for induction and regeneration of haploid wheat embryos in the wheat × maize system. *Crop Science* 39: 1722–1727.

WEDZONY M, and van LAMMEREN AAM. 1996. Pollen tube growth and early embryogenesis in wheat × maize crosses influenced by 2,4-D. *Annals of Botany* 77: 639–647.

WEDZONY M, MARCINSKA I, PONITKA A, SLUSARKIEWICZ-JARZINA A, and WOZNA J. 1998. Production of doubled haploids in *Triticale* (× *Triticosecale* Wittm) by means of crosses with maize (*Zea mays* L) using picloram and dicamba. *Plant Breeding* 117: 211–215.

ZHANG J, FRIEBE B, RAUPP WJ, HARRISON SA, and GILL BS. 1996. Wheat embryogenesis and haploid production in wheat × maize hybrids. *Euphytica* 90: 315–324.

ZHAO J, ZHOU C, and YANG HY. 2000. Isolation and in vitro culture of zygotes and central cells of *Oryza sativa. Plant Cell Reports* 19:321–326.