

The place of asymmetric somatic hybridization in wheat breeding

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Abstract Since its first development some 40 years ago, the application of the somatic hybridization technique has generated a body of hybrid plant material involving a wide combination of parental species. Until the late 1990s, the technique was ineffective in wheat, as regeneration from protoplasts was proving difficult to achieve. Since this time, however, a successful somatic hybridization protocol for wheat has been established and used to generate a substantial number of both symmetric and asymmetric somatic hybrids and derived materials, especially involving the parental combination bread wheat and tall wheatgrass (*Thinopyrum ponticum*). This review describes the current state of the art for somatic hybridization in wheat and focuses on its potential application for wheat improvement.

Keywords Asymmetric somatic hybridization · Wheat germplasm · Genomic variation · Alien introgression · Protoplast fusion

Introduction

Achieving a sustainable supply of food in the light of the inexorable rise in the global population is a major challenge for plant breeders. Crop improvement through breeding is only possible where genetic variation is present in the breeder's germplasm, but decades of intensive selection in

the major crops has inevitably narrowed their genetic base. There is therefore an urgent need to generate and/or introduce novel genetic variation, using either transgenesis, mutagenesis or wide hybridization. With respect to the latter, while conventionally viewed as involving sexual crosses (Sears 1993; Jauhar and Chibbar 1999), an alternative route is provided by somatic hybridization (Glimelius et al. 1991; Waara and Glimelius 1995; Li et al. 1999; Zhou et al. 2001a; Xia 2009). As well as not being restricted by the lack of crossability often encountered in attempts to hybridize phylogenetically distant species, somatic hybridization also offers the possibility of combining non-nuclear genomes (Bijoya et al. 1999; Zhou et al. 2001b; Cheng et al. 2004a, b; Cai et al. 2007). Following the first successful recovery of somatic hybrids in the 1970s (Carlson et al. 1972), the technique has since been applied to a range of both arable crops, including potato, tomato, tobacco, carrot, various *Brassica* spp., rice, millet, *Festuca* spp., (Melchers and Labib 1974; Melchers et al. 1978; Dudits et al. 1980; Shepard et al. 1983; Handley et al. 1986; Ozias-Akins et al. 1986; Terada et al. 1987; Sjodin and Glimelius 1989; Bauer-Weston et al. 1993; Spangenberg et al. 1995; Kisaka et al. 1998; Wang et al. 2003), and tree species, for example *Citrus* spp. and *Populus* spp. (Ohgawara et al. 1985; Park et al. 1992; Guo et al. 2000a).

Bread wheat (*Triticum aestivum*) has been under strong selection for at least 150 years. Because it was created in nature only about 10,000 years ago (Feldman et al. 1995), this selection has been applied on an already rather narrow genetic base. The species is crossable with a number of its non-cultivated and cultivated relatives, some of which have evolved attractive levels of disease resistance, stress tolerance and even end-use quality traits (Dong and Zheng 2000). A number of these traits have been successfully transferred to wheat via the sexual hybridization route

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(Zhong et al. 2002; Jauhar 2006). The process is restricted by the need to overcome both the difficulty in making a viable sexual wide hybrid and the high levels of sterility, resulting from chromosomal imbalance, which often afflict the subsequent backcross generations (Xia 2009). Furthermore, for more distant donors, the presence of the chromosome pairing inhibitor gene *Ph1* prevents recombination between the donor and the recipient wheat chromosomes (Riley and Chapman 1958). Somatic hybrids involving wheat have in the past not been achieved, mainly because of problems maintaining the regenerability of embryogenic calli or cells held in suspension. Optimization of the in vitro conditions (Xia et al. 1995) finally opened the way to establishing somatic hybridization in wheat (Xia and Chen 1996; Xia et al. 1996; Zhou et al. 1996), and since this time a growing number of somatic hybrids and derivatives have been obtained (Zhou et al. 2001a, b; Xia et al. 2003; Xiang et al. 2003a, b, 2004; Xu et al. 2003; Cheng et al. 2004a, b; Cheng and Xia 2004; Li et al. 2004; Zhou and Xia 2005; Cai et al. 2007; Cui et al. 2009). Some of these have proven to exhibit features useful in the context of wheat improvement, particularly those derived from the parental combination bread wheat with tall wheatgrass (*Thinopyrum ponticum*). The range of cytogenetic and genomic alterations induced in some of these lines has been described (Wang et al. 2004a; Peng et al. 2009; Liu et al. 2012) and the presence of donor genes established (Liu et al. 2007, 2009; Shan et al. 2008; Li et al. 2010a, b). In this review, the future of somatic hybridization in wheat and its application for wheat improvement are discussed.

Somatic hybridization between bread wheat and its related species

Somatic hybridization in the grasses was first achieved in the combination of sugarcane (*Saccharum officinarum*) and pearl millet (*Pennisetum americanum*) (Tabaeigadeh et al. 1986) and shortly thereafter in that of rice (*Oryza sativa*) with barnyard grass (*Echinochloa oryzicola*) (Terada et al. 1987), and einkorn wheat (*T. monococcum*) with pearl millet (Vasil et al. 1988). The first fertile somatic hybrids produced were obtained by fusing protoplasts of tall fescue (*Festuca arundinacea*) with those of Italian ryegrass (*Lolium multiflorum*) (Spangenberg et al. 1995). Fertile rice somatic hybrids were generated by Kisaka et al. (1998) and Liu et al. (1999). The earliest reported somatic hybrid calli involving bread wheat were recovered following electrofusion between its protoplasts and those of Italian ryegrass (Chen et al. 1992). Treating wheat protoplasts with iodoacetate facilitated the generation of a hybrid involving γ -ray irradiated naked oat (*Avena nuda*) protoplasts (Liu and

Liu 1995). The following year, hybrid plants were created following asymmetric hybridization between bread wheat and its four close relatives, tall wheatgrass, *Psathyrostachys juncea* (Xia et al. 1996), *Haynaldia villosa* (Zhou et al. 1996) and *Leymus chinensis* (Xia and Chen 1996). These hybrids produced a range of fertile derivatives, some of which were later used to determine the pattern of inheritance of key introgressed traits. In each of the four combinations, the fusion products grew more rapidly than the non-fused control protoplasts, so that the majority of the earliest formed cell clones were hybrid. In addition, unlike hybrid cells, parental protoplasts exhibited a poor level of differentiation and regeneration, perhaps reflecting a level of complementation between the two parents. The success of these combinations showed that vigorous growth and complementary effect could be used for creating somatic hybrid plants of wheat efficiently (Xia et al. 1996; Zhou et al. 1996).

Such vigorous growth and complementary effect was then used for the generation of somatic hybrids between wheat and other phylogenetically distant species. Albino plants were regenerable from the combinations bread wheat with *Bromus inermis* (Xiang et al. 1999), with *Aeluropus littoralis* (Yue et al. 2001) and with oat (*A. sativa*) (Xiang et al. 2003a, b), and green ones from the combinations involving maize (*Zea mays*) (Xu et al. 2003), Italian ryegrass (Cheng and Xia 2004), foxtail millet (*Setaria italica*) (Cheng et al. 2004b; Xiang et al. 2004), *Bupleurum scorzonerifolium* (Zhou et al. 2006), tall fescue (Cai et al. 2007) and *Arabidopsis thaliana* (Deng et al. 2007). Some of these regenerants were able to flower (Zhou et al. 2002; Xu et al. 2003; Xiang et al. 2003b; Cheng and Xia 2004) and even set viable seed (Zhou et al. 2001a; Xia et al. 2003; Cheng et al. 2004b; Cui et al. 2009). The regeneration of plants from hybrid calli derived from wide combinations typically have proven to be difficult to achieve, and plants which are regenerated are generally sterile and morphologically abnormal (Bauer-Weston et al. 1993; Louzada et al. 1993; Spangenberg et al. 1994; Begum et al. 1995). A technique to mitigate the severity of these problems is to form asymmetric hybrids, where the donor protoplasts are irradiated with UV prior to fusion; this treatment succeeds in fractionating the donor genome and has been shown to improve the chances of introgression (Sun et al. personal communication). Most of the regenerants in the examples given above used this approach.

Traits transferred via asymmetric somatic hybridization

A great deal of research effort has been devoted to using cytogenetic or genetic assays to demonstrate the hybrid

nature of regenerated somatic hybrids, but much less attention has been paid to tracking the traits transferred to or the expression of donor genes in the hybrid plants. Somatic hybrids produced from the combination potato and its nematode resistant relative *Solanum bulbocastanum* were shown to express the donor's nematode resistance (Austin et al. 1988). Similarly, resistance to infection with bacterial pathogen *Xanthomonas campestris* was successfully transferred from a *Brassica napus* line to a somatic hybrid involving *B. oleracea* as the other parent (Hansen and Earle 1995); and the root rot resistance of the trifoliolate orange (*Poncirus trifoliata*) was transferred to hybrid plants formed by fusing its protoplasts with those of the Page tangelo (*C. reticulata* × *C. paradisi*) (Guo et al. 2000b). Silique length, pod density, seed number per pod and seed color were all inherited from *Sinapis alba* in its hybrid with *B. napus* (Li et al. 2009). When wheat protoplasts were fused with UV-irradiated protoplasts of the salinity-tolerant species *Aeluropus litoralis*, the resulting hybrids showed a superior level of salinity tolerance than the parental wheat line, indicating the successful transfer of gene(s) encoding tolerance (Yue et al. 2001). Two derivatives of the somatic hybrid between the bread wheat cultivar Jinan 177 and tall wheatgrass were found to express an enhanced level of salinity tolerance, and a further six produced grain of better end-use quality than those of cv. Jinan 177 (Chen et al. 2004a; Feng et al. 2004; Liu et al. 2007, 2009). The two tall wheatgrass genes, *H11-3-3* and *H11-4-3*, which encode high molecular weight glutenin subunits (HMW-GS), were both incorporated in some of the hybrid's derivatives (Liu et al. 2007).

Somatic hybrids inherit not only the nuclear genome of both parents, but also their plastid and mitochondrial genomes. Rice somatic hybrids involving a cytoplasmic male-sterile and a male-fertile line expressed male sterility, and a DNA assay was able to show that mitochondrial DNA from the cytoplasmic male-sterile line was present in the hybrid (Bijoya et al. 1999). In most somatic hybrids described to date, the mitochondrial genomes of both parents co-exist in the hybrid or become recombined (Wolters et al. 1995; Yamagishi et al. 2002; Leino et al. 2003; Guo et al. 2007). However, the chloroplast complement tends to be inherited from just one parent in most cases, with just a few examples of recombination (Derks et al. 1991; Mohapatra et al. 1998; Liu and Deng 2000; Cardi et al. 1999). Donor mitochondrial genomic fragments have been identified in some wheat somatic hybrids (Zhou et al. 2001b; Xu et al. 2003; Cheng et al. 2004b; Cheng and Xia 2004; Li et al. 2004; Xiang et al. 2004; Cai et al. 2007), while the chloroplast complement has tended to be inherited from the wheat parent, with some evidence for co-existence and recombination (Zhou et al. 2001b; Chen et al. 2004b; Li et al. 2004; Xiang et al. 2004; Cai et al. 2007).

Cytogenetic analysis of asymmetric somatic wheat hybrids

Genomic in situ hybridization (GISH) (Parokony et al. 1992) is a highly informative method of identifying and characterizing donor chromosomes or chromosome fragments present in somatic hybrids (Zhou et al. 2001b; Wang et al. 2005). GISH analysis of somatic hybrid regenerants formed from the combinations of bread wheat with either *H. villosa* (Xia et al. 1998; Zhou et al. 2001a, b), oat (Xiang et al. 2003a) or *S. italica* (Xiang et al. 2004) identified only few donor chromosomes and chromosome fragments, while no complete donor chromosomes were seen in the derivatives of combinations made between bread wheat and tall wheatgrass (Xia et al. 2003; Chen et al. 2004a; Wang et al. 2005), maize (Xu et al. 2003), *P. juncea* (Li et al. 2004), Italian ryegrass (Cheng and Xia 2004), *B. scorzonnerifolium* (Zhou et al. 2006) or tall fescue (Cai et al. 2007). Varying the pre-fusion irradiation dosage given to the donor protoplasts proved effective for controlling the amount of donor genome transferred (Xiang et al. 2003b; Cheng et al. 2004b, Zhou and Xia 2005; Cui et al. 2009). A GISH analysis of five derivatives of a wheat/tall wheatgrass hybrid identified the presence of donor chromosome fragments scattered over between four and eight of the hybrid's chromosomes (Chen et al. 2004b; Wang et al. 2004a). The mitotic chromosome number, as measured in root tip cells, was very stable over seven generations of self-fertilization, and there was an indication of minor variation in arm ratio and the length of some chromosomes compared to the standard bread wheat karyotype. At meiosis, the lines behaved indistinguishably from the parental bread wheat cultivar, producing >90 % ring bivalents and only few univalents (Wang et al. 2004a; unpublished data). The implication is that the introgressed tall wheatgrass chromosomal segments were too small to have any appreciable effect on chromosome pairing. Analyses based on DNA profiling indicated that the donor DNA was stably inherited through meiosis. Stability at the cytogenetic and DNA level was mirrored in the observed phenotypic stability of the various asymmetric somatic hybrid derivatives (Xia et al. 2003; Chen et al. 2004a; Wang et al. 2004a; Xia 2009).

The inheritance of traits of breeding value

The derivatives of asymmetric somatic hybrids formed between bread wheat cv. JN177 and tall wheatgrass have displayed a wide range of phenotypic variation, including height, tiller number, spike length, awn length, grain size, grain number per spike, stress tolerance, disease resistance, grain protein content and flour quality. Some have

performed better than the parental cultivar with respect to grain yield, salinity tolerance, drought tolerance, disease rust resistance, dwarfism, grain size and end-use quality (Xia et al. 2003; Feng et al. 2004; Zhang et al. 2005; Liu et al. 2007; Xia 2009). Selected lines have been used as parental material in a conventional breeding program, which has led to the release of the cultivar SR3, adapted to cultivation in salinity-affected soils. Five phenotypic classes of derivative were recognized. Type I lines feature a large square spike which sets >50 grains (the parental cultivar spike sets about 40 grains). In trials carried out over 3 years in Shandong Province, the yield of line SR1 reached 10.3 t ha⁻¹, allowing this selection to be registered in China as a high yielding cultivar. Type II lines expressed an enhanced tolerance to drought and salinity; when grown under drought-affected conditions, the yield of one selection was nearly 7 t ha⁻¹ or >11 % more than that of the control cv. Lumai 23. cv. SR3, a second selection in this group, was noticeably more salinity tolerant than cv. JN177. Its yield reached 6.3 t ha⁻¹ (~12 % more than the control cultivar Dekang 961) in 0.4 % saline soils. The germinability of cv. SR3 was 77 % when imbibed in 2 % w/v NaCl, while in cv. Dekang 961 the level was only 35 %. As a result, cv. SR3 has recently been registered for commercial cultivation in Shandong Province and has been grown over some 433 kca since its registration. Another selection from the same somatic hybridization has (SR14) shown notable levels of resistance to both soil salinity and alkalinity and is currently being trialed in the field. The third phenotypic group was characterized by a small, awnless spike and was of interest because of the expression of immunity to both yellow rust and powdery mildew. Type IV lines were shorter and tillered more profusely than cv. JN177 and their flour had an SDS sedimentation value predictive of superior rheological quality (Liu et al. 2006, 2009). Their grain protein content was about 20 % and frequently contained unusual HMW-GS combinations (Liu et al. 2007, 2009). Finally, type V lines produced long spikes and large grains. Their awns were also longer and their leaves longer and broader than those of cv. JN177.

Genetic and epigenetic variation induced by somatic hybridization

It is widely understood that the fusing of genomes within a single nucleus can trigger genomic shock, as seen in de novo polyploids involving *Arabidopsis* spp. (Comai 2000; Madlung et al. 2002; Wang et al. 2004b), *Brassica* spp. (Song et al. 1995), *Spartina* spp. (Salmon et al. 2005), *Tragopogon* spp. (Tate et al. 2006) and certain Triticeae spp. (Ozkan et al. 2001; Shaked et al. 2001; Han et al. 2003; He et al. 2003; Ma et al. 2004; Feldman and Levy

2005). Evidence that genetic and epigenetic variation was induced by somatic hybridization suggests that one or the other (or both together) largely explains the phenotypic diversity of the somatic hybrid derivatives. Comparison of the microsatellite profiles of six hybrid introgression lines produced from the wheat/tall wheatgrass somatic hybrid showed that novel alleles (mostly involving a change in the number of microsatellite repeats) were present at about one in three loci. Two DNA profiling assays based on transposons (IRAP and REMAP, Kalendar et al. 1999) indicated that about 6.5 % of the fragments amplified differed in length between cv. JN177 and the somatic hybrid derivatives, suggesting a burst of transposition during the somatic hybridization; at least one such de novo transposition event occurred in the vicinity of a gene encoding an endosperm storage protein (Liu et al. 2009). AFLP (amplified fragment length polymorphism) fingerprinting has demonstrated that although some novel fragments have been generated, most of the alterations induced in the two somatic hybrid products have involved fragment loss (Cui et al. 2009), the same as is the case in newly made sexual allopolyploids (Ozkan et al. 2001). It has been suggested that sequence elimination during allopolyploidization provides a physical basis for the diploid-like meiotic behavior of newly formed allopolyploids (Ozkan et al. 2001). Thus, the meiotic pressure might also be responsible for these sequence losses during somatic hybridization. There was also evidence for the induction of epigenetic variation in the somatic hybrids. In plants derived from the somatic hybrid between wheat and tall fescue, the global genomic methylation level lay in the range 33.5–34.8 %, significantly higher than what prevailed in either parent (31.9 % in the recipient and 25.6 % in the donor) (Cai et al. 2007).

Novel allele and gene expression in somatic hybrid lines

A comparison between the HMW-GS gene sequences present in the *II-12* and the relative introgression lines and those present in the parental lines has shown that in addition to the two inherited from the donor parent, six derived from point mutations to cv. JN177 parental genes, ten probably resulted from unequal crossing over or DNA replication errors within the wheat HMW-GS sequences, three appeared to have been activated by transposon movement and one probably arose via recombination between two parental genes (Feng et al. 2004; Liu et al. 2007, 2009). A further three novel HMW-GS genes have been identified in derivatives of a symmetric wheat/tall wheatgrass somatic hybrid (Gao et al. 2010). Novel alleles at genes encoding both low molecular weight glutenin subunits and gliadins were also revealed by Chen et al. (2008a, b, 2009). The non-parental alleles present appear to

have arisen from one or more of point mutation, deletion/duplication of blocks of repeats, gene shuffling and transposition (Liu et al. 2007, 2009). Some of these novel endosperm storage protein genes have since been over-expressed in wheat, where they appear to have a beneficial effect on end-use quality (Chen FG, personal communication).

Given the enhanced salinity tolerance of cv. SR3, efforts were made to track the transcription and expression of a number of salinity tolerance-related genes (Shan et al. 2004; Wang et al. 2008; Peng et al. 2009; Li et al. 2010a, b; Liu et al. 2012). Microarray analysis uncovered that the R2R3-type MYB gene *TaMYB73* was induced by treating plants with either NaCl or one of several phytohormones, or by the withholding of water. The peptide sequence of the product of the cv. SR3 allele differed by several residues from that of the cv. JN177 product. When heterologously expressed in *A. thaliana* it was able to enhance the plant's tolerance to NaCl, LiCl or KCl (He et al. 2012). When the cv. SR3 and cv. JN177 coding sequences of 18 *TaWRKY* genes were compared, it was revealed that ten of the sequences were non-identical, with most of the variation due to point mutations (15 transitions and six transversions); however only four of the variants implied any change in the product's peptide sequence. Nine of the 18 genes were up-regulated by the provision of the phytohormone salicylic acid, while six were up-regulated by salinity, drought stress or exposure to the phytohormone abscisic acid (ABA) (Zhu et al. 2013). The constitutive expression in *A. thaliana* of *TaWRKY71-1* resulted in the formation of hyponastic leaves (Qin et al. 2013).

Among other genes differentially transcribed in cv. SR3 in response to salinity were *RHD3* (root hair defective) and *TaCHP* (encoding a cysteine, histidine and proline-rich zinc finger protein), both of which were down-regulated (Shan et al. 2005; Li et al. 2010a). In the latter case, transcript abundance was higher in cv. SR3 than in cv. JN177, and was also suppressed by both the imposition of drought stress and exposure to ABA. The constitutive expression of *TaCHP* in both bread wheat and *A. thaliana* improved the level of salinity tolerance (Li et al. 2010a). The use of the suppression subtractive hybridization method led to the detection of *WRSI5*, a salinity-responsive gene which encodes a Bowman–Birk domain-type protease inhibitor (Shan et al. 2008). Its transcript abundance was increased in the roots of cv. SR3 plants subjected to salinity, drought or oxidative stress. The gene was shown to be induced in the endodermal cells of the mature region of the cv. SR3, but not of the cv. JN177 root tip. When constitutively expressed in *A. thaliana*, it improved seedling salinity tolerance (Shan et al. 2008). When the salinity stress up-regulated gene *TaDi19A* was heterologously expressed in *A. thaliana*, the level of sensitivity to salinity,

ABA and the osmotic stress agent mannitol was increased (Li et al. 2010b). *TaOPRI*, a gene encoding a 12-oxo-phytodienoic acid reductase, was inducible by salinity stress in cv. SR3 and was transcribed more abundantly in an ABA-dependent manner in the seedling root of cv. SR3 than in those of cv. JN177. The constitutive expression of *TaOPRI* significantly enhanced the level of salinity tolerance, while its heterologous expression in *A. thaliana* buffered root growth from damage caused by salinity and certain oxidants, as well as raising the level of the plant's sensitivity to ABA (Dong et al. 2013). Although no sequence differences were known between the cv. SR3 and JN177 copies of any these salinity tolerant genes, their differential transcription may well be epigenetically regulated.

Concluding remarks

Asymmetric somatic hybridization has been established as a viable chromosome engineering technique in wheat, with potentially rewarding applications in wheat improvement. It has some specific advantages over the more conventional route of sexual hybridization, namely (1) its use is not restricted by sexual incompatibility, (2) it tends to introgress multiple short donor chromosome fragments, rather than whole chromosomes or chromosome arms, (3) it allows the exploitation of non-nuclear DNA from the donor, (4) the process is more rapid than sexual hybridization, as less subsequent crossing is needed to restore fertility and a normal somatic chromosome complement and (5) it induces a range of both genetic and epigenetic variants. Overall, somatic hybridization provides a more flexible means to introgress genes from related species than sexual wide crossing and has considerable potential as a technique for widening the gene pool of crop species such as wheat.

Although a number of somatic hybrid plants and derivatives have by now been obtained, the process remains difficult in wheat. A major technical issue is to maintain regenerable embryogenic calli or cells in suspension as a source of suitable protoplasts; the suitability appears to be genotype dependent (Xia et al. 1995). Our successful optimization of the fusion and culture protocols (Xia et al. 1995, 1996) has led to the development of a “three-cell” system. Here, the recipient material is a mixture of protoplasts obtained from a long-term suspension culture which are able to divide freely, along with those obtained from de novo produced embryogenic calli (Xiang et al. 2003a, b, 2004; Xu et al. 2003; Cheng and Xia 2004; Cheng et al. 2004a, b; Li et al. 2004; Cai et al. 2007; Deng et al. 2007). A description of the genomic and cytogenetic properties of the three-cell system has been

given by Xiang et al. (2010). A second technical issue relates to the poor self-fertility of many of the somatic hybrids, although the use of an asymmetric hybridization strategy mitigates the severity of this problem by minimizing the size of the donor fragments (Xia 2009). In general, as is also the case for sexually produced wide hybrids, the closer the phylogenetic relationship between the donor and the recipient, the better is the chance of recovering self-fertility (Zhou et al. 2001a; Xia et al. 2003; Cheng et al. 2004b; Cui et al. 2009).

We have observed that the performance of independent selections made from the same somatic hybrid combination can differ with respect to a number of agronomic traits. While superior performance with respect to breeders' traits such as end-use quality, drought tolerance or yield provides the basis for crop improvement, it is also of interest in the context of unraveling the genetic basis of phenotype. The productivity, salinity tolerance and drought tolerance of the somatic hybridization-derived cv. SR3 are all higher than those of its parental cultivar JN177. The recent demonstration that the gene *Tasro1* is differentially transcribed in cv. SR3 has suggested that this gene plays an important part in the cultivar's superior salinity tolerance and vigor. A better understanding of the relative contributions of genetic and epigenetic variation to the phenotype of somatic hybrid introgression lines could open up novel opportunities in the ongoing efforts to improve wheat and other crops.

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Conflict of interest The authors declare that they have no conflict of interest.

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