Split-gene system for hybrid wheat seed production

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Hybrid wheat plants are superior in yield and growth characteristics compared with their homozygous parents. The commercial production of wheat hybrids is difficult because of the inbreeding nature of wheat and the lack of a practical fertility control that enforces outcrossing. We describe a hybrid wheat system that relies on the expression of a phytotoxic barnase and provides for male sterility. The barnase coding information is divided and distributed at two loci that are located on allelic positions of the host chromosome and are therefore “linked in repulsion.” Functional complementation of the loci is achieved through coexpression of the barnase fragments and intein-mediated ligation of the barnase protein fragments. This system allows for growth and maintenance of male-sterile female crossing partners, whereas the hybrids are fertile. The technology does not require fertility restorers and is based solely on the genetic modification of the female crossing partner.

hybrid wheat breeding | split-gene approach | site-specific recombination | intein-mediated protein splicing

Hybrids often display increased yield, enhanced yield stability, and improved abiotic and biotic stress resistance that result from heterosis (hybrid vigor) (1). However, hybrid seeds cannot be saved without a significant loss in performance, a fact that protects the intellectual property of seed companies. The benefits of hybrid systems are countered by the increased seed production costs that result from complex breeding schemes. Therefore, there is a strong incentive to develop systems that facilitate hybrid production, particularly in crops where hybrid production has been impossible or difficult to develop.

Hybrid plants are produced by cross-pollination of genetically diverse parental lines. Several prerequisites must be fulfilled for commercial hybrid seed production. First, for self-pollinating (autogamous) species, self-pollination of the “female” must be precluded to ensure that the plant will only be cross-pollinated by the chosen crossing partner (i.e., the “male” line) (2). Second, because male-sterile plants cannot be multiplied by selfing, technical solutions must be available for the multiplication of the sterile crossing partner (3). Third, in crops for which seeds are the harvest product (e.g., all cereals), sterility must be overcome in the hybrid generation to obtain a full yield in the commercialized product (2).

The most efficient way to promote cross-pollination in bisexual plants is male sterility, which can be achieved by various means. In maize, which is the world’s most valuable hybrid, the female crossing partner can be sterilized by manual detasselling (removal of the anthers) (4); however, the detasselling technique cannot be applied to crop plants that do not have a physical separation of the male and female flower parts. The most well-established genetic system is cytoplasmic male sterility (CMS), which is based on mutated mitochondrial DNA (5). The effect of the male sterility-inducing cytoplasm can be neutralized by fertility restorers (i.e., nuclear genes that are specific to each CMS system). CMS is reasonable for hybrid breeding only if mutants for cytoplasmic sterility and fertility restoration are available in a given crop, which renders CMS a relatively inflexible system. The introgression of CMS mutants is complex, because the different genetic components have to be discovered and brought

together separately. CMS is functional in important cereals, such as rice and rye; however, in hexaploid wheat, CMS is difficult to develop, complex to maintain, and only marginally reliable. In particular, reliable restorer genes are not yet available, making F_1 fertility restoration difficult. The lack of availability of restorer genes has been a major factor in limiting the application of CMS to wheat (6, 7). Moreover, CMS systems are sensitive to environmental factors, such as temperature and photoperiod (8). As a result, no wheat CMS system with more than a regional application is currently used in hybrid seed production (9).

All of the hybrid cultivars that are registered for the European wheat market were produced by sterilizing the pollen recipient through the application of a chemical hybridization agent (CHA) (gametocide) (10). The plant growth regulator Croisor 100 (sintofen; formerly Dupont-Hybrinova, Saaten Union Recherche) is currently the only wheat CHA that is used for commercial European hybrid wheat production (www.hybridwheat.net). Sterile females are grown in alternating stripes in close proximity to untreated plants that display good pollen shedding properties, and the hybrid seed is harvested from the females after cross-pollination (2, 9). However, the commercial deployment of CHAs is hampered by several problems, including compromised seed production in wheat females that are treated with CHAs (11) and narrow application windows attributable to prevailing environmental factors, such as rain, wind, and heat (10, 12). Thus, the use of CHAs bears a substantial financial risk.

There have been major efforts to deploy transgenic technology for hybrid systems (2); however, Seedlink from Bayer CropScience is the only commercialized transgenic male-sterility system. The Seedlink method relies on the expression of a cytotoxic

Significance

Global food security demands the development of new technologies to increase and secure cereal production on finite arable land without increasing water and fertilizer use. Although the use of heterosis through hybrid breeding has produced tremendous economic benefits in worldwide crop production, less than 1% of the global wheat area is planted with hybrids. One of the greatest bottlenecks in breeding hybrid wheat is the lack of an efficient sterility system to block self-pollination. This report describes a recessive system for pollination control in wheat. We demonstrate the implementation and feasibility of the system for generation and maintenance of the male-sterile parent, hybrid seed production and full restoration of fertility in the hybrid wheat seed.

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ribonuclease from *Bacillus amyloliquefaciens* (barnase) in the male reproductive tissue tapetum, which is essential for pollen development (13). The male-sterile line is maintained by backcrossing with a nontransgenic plant, which results in a mixed population comprising 50% sterile plants. By linking glufosinate resistance (LibertyLink) to the *barnase* transgene, male-sterile plants can be selected. Fertility restoration in the F₁ hybrid progeny results from the expression of the *barstar* gene, which is delivered by the father. Barstar counteracts barnase by forming a specific and stable one-to-one protein complex (14). Although this method is used successfully for hybrid canola production, the dominant dual-component system requires the expression of transgenes in each crossing partner, which involves additional breeding efforts.

Here, we describe a recessive “split-gene” system that has the potential to overcome several difficulties associated with the systems that are currently used. Our system relies on the expression of a *barnase* gene that is encoded by two nonoverlapping sequences at complementary genetic loci (15). Following translation, the barnase fragments are assembled into an active phytotoxic enzyme via an autocatalytic process of intein-mediated *trans*-splicing (16, 17). The tapetum is destroyed after the phytotoxic barnase is formed, which prevents the formation of viable pollen. Both *barnase* gene fragments reside at identical chromosomal positions on physically distinct homologous chromosomes (“isoloci”). These partial genes functionally mimic a pair of recessive alleles because a plant will produce active barnase and exhibit a male-sterile phenotype only as a “heterozygote” (i.e., when the plant carries both complementary “alleles”). These plants can then be used as the female (male-sterile) crossing partner in hybrid breeding. During meiosis, the members of a pair of *barnase* loci segregate into separate gametes. Consequently, the hybrid progeny inherit only one of the complementary fragments after pollination, so they remain fertile. This genetic design facilitates a mode of fertility restoration that should be robust. Moreover, because only the female crossing partner is genetically manipulated, this hybridization system is more flexible to use than are systems that also require a genetically modified male parent.

Wheat is one of the most important staple crops. Research has underscored that the implementation of hybrid wheat breeding has the potential to achieve breakthrough increases in yield, yield stability, and sturdiness against biotic and abiotic stresses. Recent large-scale phenotyping involving extensive collections of inbred lines and hybrids revealed that hybrids were superior to

the mean of their parents for grain yield, susceptibility to frost, leaf rust, and *Septoria tritici* blotch (18, 19). A consistently higher grain yield stability for wheat hybrids compared with lines was observed in multilocation field trials (20). The yield benefits and the robustness of hybrids may provide additional benefits related to the future climatic changes that are predicted to impair crop production (21). Therefore, the development of technologies that enable the benefits of heterosis to be captured in a major crop, such as wheat, is of paramount importance.

Results

Description of the Vector Constructs and the Experimental Strategy.

The cloning of the barnase-containing “provectors” used in this study has been described previously (22). The vectors that contain nonoverlapping fragments of a *barnase* gene from *Bacillus amyloliquefaciens* are fused to the N- and C-terminal sequences of an intein that is derived from the *Synechocystis* sp. *DnaB* helicase gene (Fig. 1A). For tapetum-specific expression, the *barnase*–intein fusion is controlled by the rice *osg6B* promoter (23). Upon translation, the inteins covalently fuse the barnase fragments in an autocatalytic reaction to produce an intact cytotoxic barnase protein (Fig. 1D), which leads to male sterility.

The localization of both *barnase* gene fragments to the same locus on homologous host chromosomes is achieved by transforming plants with a construct that contains both genetic elements (the provector). Next, the primary prolocus is derivatized by using site-specific deletions. For this purpose, the provectors contain *attB* and *attP* recombination sites to serve as targets for ϕ CH31 integrase (a site-specific recombinase) (24, 25). Site-specific recombination is induced by crossing a plant that carries the prolocus with a plant that carries the ϕ CH13130 locus as the integrase source (Fig. 1B) (26). Site-specific deletions may lead to two different loci that contain only the N- or the C-terminal fragment of the barnase (hereafter referred to as derivative loci “A₁” and “A₂”; Fig. 1C). In the case of only an N-terminal fragment, a hybrid *attL* sequence is produced via a reaction between *attB* and *attP*₂; in the case of only a C-terminal fragment, a reaction between *attP*₁ and *attB* creates a hybrid *attR* sequence. *attL* and *attR* are not substrates for the ϕ CH31 integrase in plants. Consequently, the described ϕ CH31 integrase-mediated recombination reactions are irreversible, and the two reactions are mutually exclusive.

Fig. 2 describes the different experimental steps required to develop the system including the predicted genotypes and phenotypes.

Fig. 1. Vector constructs used in this study. (A and B) The genetic structure of the *barnase* expression vector (“pro-vector”) (A) and the ϕ CH31-integrase-expression vector (B). The provector harbors *attP* and *attB* sequences that serve as targets for the site-specific recombinase ϕ CH31. Note that only the T-DNA part of the vectors is illustrated (not drawn to scale). (C) Phage ϕ CH31 integrase-mediated site-specific recombination results in the derivative loci A₁ and A₂. Recombination results in the hybrid products *attR* and *attL*. (D) A schematic illustration of the assembly of active barnase cytotoxin via the intein-mediated *trans*-splicing of two precursor molecules. Bar-N and Bar-C, N- and C-terminal gene fragments of the *Bacillus amyloliquefaciens* *barnase* gene; HPTII, hygromycin phosphotransferase; IntN and IntC, N- and C-terminal intein sequences from the *DnaB* gene of *Synechocystis* sp.; LB and RB, T-DNA left and right borders; ocs, octopine synthase terminator; ϕ CH31, phage ϕ CH31 recombinase coding sequence; PSK, intron PSK7-13 from *Petunia hybrida*; Ptap, tapetum-specific *osg6B* promoter from rice (23); Pubi, maize ubiquitin 1 promoter; S, (GGGS)₃ flexible peptide linker; UBQ, intron UBQ10-11 from *Arabidopsis thaliana*.

Fig. 2. Experimental design for the production of hybrid seed. Genotypes that have an expected male-sterile phenotype are symbolized in blue. (A) The generation of the male-sterile parent for hybrid breeding. After transforming the provector, the primary transformants (T_0) are expected to be male-sterile as a result of barnase expression. To ensure that the phenotype is stable, and to create more target plants for recombination, the T_0 plants are backcrossed. In the event of a single-copy integration of the prolocus, the T_1 progeny are expected to segregate so that ~50% of the plants are male-sterile (transgenic) and 50% are fertile (null-segregants). In the next step, the sterile T_1 plants are crossed with plants that express a phiC31 site-specific recombinase (encoded by pICH13130). In the developing T_2 plants, the integrase may catalyze the site-specific deletions between the *attP* and *attB* sites of the prolocus. Two alternative reactions can lead to the deletion of either the C-terminal or the N-terminal part of the locus, which results in the formation of the derivative loci A_1 or A_2 . The derivative loci will naturally reside at exactly the same genetic position on two homologous chromosomes (because they originate from one prolocus). Depending on the time point at which recombination occurs, the F_2 plants may be genetic chimera that contain recombined and nonrecombined sectors. Plants carrying only A_1 or A_2 should be fertile, because no complete barnase protein can be produced. The A_1 and A_2 lines are then crossed (either as T_3 plants or T_4 plants after one round of self-pollination), and a portion of the resulting progeny is expected to carry both the A_1 and A_2 loci (25% of the progeny if both parents are hemizygous for A_1 and A_2 , and 50% or 100% of the progeny if one or both parents are homozygous for A_1 or A_2). These heterozygous genetic segregants are expected to be male-sterile because of the coexpression of barnase from the A_1 and A_2 loci. (B) A strategy for maintaining the male-sterile line. Reproduction of the heterozygous male-sterile line A_1A_2 can be accomplished by crossing such lines with a homozygous fertile “maintainer” line, A_1A_1 or A_2A_2 . The progeny of this cross may segregate the seeds so that 50% will have the genotype A_1A_2 (like the female parent) and, depending on the male parental line that was used, 50% will have the genotype A_1A_1 or A_2A_2 . (C) Hybrid seed production. Male-sterile plants with the genotype A_1A_2 are used as the female crossing partners for hybrid seed production. The allelic position of the complementary barnase fragments enforces 100% segregation during meiosis. This results in male fertility and seed set in the hybrid progeny, because all segregants carry only an inactive barnase fragment (either A_1 or A_2). BC, backcrossing step; S, self-pollination step.

Generation of the A_1 and A_2 Loci. A total of 180 sterile primary transformants (T_0) carrying the prolocus were produced by genetic transformation and were backcrossed to wild-type plants (Fig. 2A). More than 95% of the backcrosses led to vital seeds, which demonstrates that sterility was restricted to the male gametes and did not affect fertility in general. For further analysis, we preferentially selected lines that carried a single barnase locus (indicated by a 1:1 segregation of transgenic sterile vs. nontransgenic fertile T_1 plants; assayed using fertility assays and Southern blot analyses).

To induce deletions at the prolocus, 96 male-sterile T_1 plants (derived from 46 independent T_0 plants) were crossed with doubled-haploid (DH) plants that expressed an active phiC31 integrase recombinase (Fig. 2A). The genotypes of 862 fertile T_2 plants were screened for the occurrence of the A_1 and A_2 loci by performing PCRs that amplified the barnase-intein regions of the A_1 and A_2 loci (Table S1 and Fig. S1). As expected, all plants

carried the integrase transgene. A total of 56% (482 plants) harbored the barnase locus; however, the loss of the barnase locus through segregation was expected for a proportion of the T_2 plants because the T_1 generation is hemizygous for the target loci. To confirm the site-specific recombination, PCRs were conducted to amplify the “excision empty donor site” of the derivative A_1 and A_2 loci, including the corresponding hybrid sequences *attL* or *attR* (Fig. S1). The presence of *attL* and *attR* was confirmed by sequencing the PCR products. A total of 97% (467 plants) of the cotransgenic T_2 plants harbored recombinant loci. Of these plants, 78% (365 plants) carried both A_1 and A_2 loci. We identified 102 T_2 plants (derived from 24 independent lines) that carried only an A_1 or A_2 locus. All of these hemizygous segregants displayed a fertile phenotype.

Thus, we conclude that the switch from a sterile phenotype in the T_0 and T_1 plants into a fertile phenotype in the T_2 plants

Fig. 3. Phenotype of the female crossing partner and the hybrid F₁. (A) The morphology of the ears from the female parent that carries both isoloci 7031- A_1 and 7031- A_2 . The ears display the typical “open floret” phenotype and contain no seeds. Other ears of these plants were used as pollen acceptors for the production of hybrid F₁ plants (B). All hybrid progeny plants displayed full fertility and carry either A_1 or A_2 .

A_2 . Transformation of the primary constructs was performed using particle bombardment. In the majority (78%) of T₂ plants that displayed recombination, the loci A_1 and A_2 were not separated. Therefore, we conclude that most of the primary transformants carried rather complex DNA integrations that may have been resolved into unpredictable products by single or multiple consecutive phiC31 recombination events. In the future, *Agrobacterium*-mediated transformation may be applied to produce less complex events that segregate more A_1 and A_2 isoloci following recombinase-mediated separation, thus making the system development more efficient. An alternative approach may be to transform constructs carrying only A_1 and A_2 independently, followed by screening for closely integrated events that display an acceptable rate of recombination between the two loci. This approach has the advantage of saving the integrase-mediated recombination step; however, it is compromised by the requirement of screening a large number of integration events and by the incomplete repulsion of the events.

Importantly, to apply the split-gene system to other wheat varieties, the A_1 and A_2 loci are not required to be developed de novo. Rather, using standard marker-assisted breeding, line conversion may be accomplished by repetitive backcrossing of the A_1 and A_2 loci independently.

The genetic design of the male-sterile female line provides the opportunity for maintaining the sterile lines, which is a prerequisite for a future commercial application of this technique. Crossing the heterozygous female line (A_1A_2) with a homozygous fertile “maintainer” line (e.g., A_1A_1 or A_2A_2) results in ~50% of the male-sterile progeny having the female genotype. In future approaches, linking the isolocus that is not present in the maintainer line to a transgene conferring herbicide resistance may enable the selection of the female genotype A_1A_2 . However, this in-field production of the male-sterile lines would require overplanting and eliminating half of the sown plants to attain a pure stand of male-sterile female plants on the stripe-breeding

production field. Alternatively, the female A_1A_2 line may be produced by crossing homozygous A_1A_1 and A_2A_2 lines. This cross would result in 100% male-sterile plants (A_1A_2). For this purpose, CHAs may be used to sterilize either the A_1A_1 line or the A_2A_2 line in small-scale applications. The resulting pure, sterile populations would enable the mixed planting of male and female parental lines in the production field, a strategy that would improve the economics of hybrid wheat seed production because almost the entire area could be used for hybrid seed harvesting (32). The use of CHAs on a small scale is, however, technically difficult.

Recently, several other transgenic systems were suggested in which male sterility was induced by external stimulation of an otherwise fertile plant (“conditional male-sterility systems”). Syngenta generated tobacco plants that expressed a modified form of D-amino acid oxidase (DAAO) in the tapetal cells (33). When D-glufosinate, the nonphytotoxic enantiomer of the commercial herbicide glufosinate, is sprayed as a “protoxin,” DAAO oxidizes D-glufosinate to 2-oxo-4-(methylphosphinyl)-butanoic acid; phytotoxic L-glufosinate is subsequently formed via intermediates. This compound causes tapetal cell death, which in turn induces male sterility. In a system that has been used by Monsanto, the expression of a 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), which confers herbicide tolerance, is specifically inhibited in tapetal cells by either miRNA-mediated translational inhibition or transcriptional repression (34, 35). Thus, cells that are necessary for pollen development are specifically unprotected against the phytotoxic effects of glyphosate and are killed during herbicide treatment. However, the benefits of conditional male sterility are compromised by the requirement for the large-scale spraying of chemicals onto production fields, resulting in increased costs and short biological application windows similar to classical CHAs.

Compared with line varieties, hybrid wheat currently occupies only a small niche in the market. Less than 1% of the global wheat area is planted with hybrids (9). Apart from a cost-effective

hybridization system, there are several biological bottlenecks that hamper the implementation of a cost-efficient hybrid seed production system. Because the floral biology of most wheat varieties is ill-adapted to cross-fertilization (29), a “re-designing” of the cleistogamic wheat flower has been suggested (7). Ideally, both male and female parental plants would possess open flowering spikelets. For optimal hybrid seed production, the sterile females must open their flowers simultaneously with those of the male crossing partners, which must release much long-lived viable pollen with good aerodynamics outside the floret. However, wheat produces only a comparably low level of pollen that settles quickly because it is relatively heavy and is viable only for a relatively short period (36).

Finally, because the heterosis of a hybrid may increase with genetic divergence between the parents, it will be of importance to group wheat lines into genetically divergent heterotic pools that combine the maximum use of yield heterosis with a high end-user quality (9). Currently, no divergent “heterotic pools” exist for wheat.

This report describes the successful implementation of a transgenic approach for hybrid wheat production. However, whether the prospects of the split-gene system will be translated into commercial hybrid wheat breeding and products in the future remains open. Ultimately, the successful realization of any genetically modified organism-based technology for agriculture will depend on industrial and consumer acceptance and on international trade regulations (37). Despite these issues, we hope that the split-gene system described here will be a valuable future contribution to unblocking bottlenecks associated with hybrid wheat breeding.

Materials and Methods

Transgenic Wheat Plants. Spring wheat (*Triticum aestivum* L. cultivar Bob-white) was used throughout this study. The plants were grown under controlled greenhouse conditions with 16 h of light at 20 °C and 8 h of darkness at 16 °C. The provectors were transformed using biolistic particle bombardment using a modified protocol that has been described previously (38).

Transgenic calli were selected on a medium containing 100 mg/L hygromycin B (Roche). Transgenic plantlets were selected in a medium containing 50 mg/L hygromycin B. For maintenance and propagation, male-sterile plants were pollinated by placing pollen-shedding anthers of untransformed plants into the closed flower. Plants were propagated by embryo rescue on half strength Murashige and Skoog medium, (M0222, Duchefa) supplemented with 15 g/L sucrose, 0.1 g/L casein hydrolysate, 0.2 mg/L copper(II) sulfate pentahydrate, 0.5 mg/L thiamine, and 50 mg/L myo-inositol.

Molecular Analyses of the Transgenic Plants. The experimental strategies used for polymerase chain reactions and DNA gel blot analyses are detailed in Table S2 and Fig. S1.

Fertility Assays. Fertility was evaluated by determining the occurrence of seeds on mature spikes. Alternatively, to determine fertility before manual pollination, the amount and vitality of the pollen was assayed. For this purpose, pollen from two flowers of two to seven ears from each plant was stained with Alexander stain (39) and examined under a light microscope. The assays were performed 2–3 d before anthesis.

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- Schnable PS, Springer NM (2013) Progress toward understanding heterosis in crop plants. *Annu Rev Plant Biol* 64:71–88.
- Kempe K, Gils M (2011) Pollination control technologies for hybrid breeding. *Plant Breed* 130(4):417–437.
- Perez-Prat E, van Lookeren Campagne MM (2002) Hybrid seed production and the challenge of propagating male-sterile plants. *Trends Plant Sci* 7(5):199–203.
- Smith CW, Betrán J, Runge E (2004) *Corn: Origin, History, Technology, and Production* (Wiley, Hoboken, NJ).
- Chen L, Liu Y-G (2014) Male sterility and fertility restoration in crops. *Annu Rev Plant Biol* 65:5.1–5.28.
- Angus WJ (2001) United Kingdom wheat pool. *The World Wheat Book*, eds Bonjean AP, Angus WJ (Lavoisier, Paris), pp 103–125.
- Whitford R, et al. (2013) Hybrid breeding in wheat: Technologies to improve hybrid wheat seed production. *J Exp Bot* 64(18):5411–5428.
- Kaul MLH (1988) *Male Sterility in Higher Plants* (Springer, Berlin, Heidelberg).
- Longin CF, et al. (2012) Hybrid breeding in autogamous cereals. *Theor Appl Genet* 125(6):1087–1096.
- Cisar G, Cooper D (2002) Hybrid wheat. *Bread Wheat: Improvement and Production*, eds Curtis BC, Rajaram S, Gomez Macpherson H (Food and Agriculture Organization, Rome), pp 157–174.
- Adugna A, Nanda GS, Singh K, Bains NS (2004) A comparison of cytoplasmic and chemically-induced male sterility systems for hybrid seed production in wheat (*Triticum aestivum* L.). *Euphytica* 135(3):297–304.
- Pickett AA (1993) Hybrid wheat—results and problems. *Advances in Plant Breeding* (Paul Parey Scientific, Berlin), Vol 15.
- Mariani C, De Beuckeleer M, Truettner J, Leemans J, Goldberg RB (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. *Nature* 374(6295):737–741.
- Mariani CV, et al. (1992) A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. *Nature* 357(6377):384–387.
- Gils M, et al. (2008) A novel hybrid seed system for plants. *Plant Biotechnol J* 6(3):226–235.
- Saleh L, Perler FB (2006) Protein splicing in cis and in trans. *Chem Rec* 6(4):183–193.
- Perler FB (1998) Protein splicing of inteins and hedgehog autoproteolysis: Structure, function, and evolution. *Cell* 92(1):1–4.
- Gowda M, Longin CFH, Lein V, Reif JC (2012) Relevance of specific versus general combining ability in winter wheat. *Crop Sci* 52(6):2494–2500.
- Longin CFH, et al. (2013) Hybrid wheat: Quantitative genetic parameters and consequences for the design of breeding programs. *Theor Appl Genet* 126(11):2791–2801.
- Mühleisen J, Piepho HP, Maurer HP, Longin CFH, Reif JC (2014) Yield stability of hybrids versus lines in wheat, barley, and triticale. *Theor Appl Genet* 127(2):309–316.
- de Vries GE (2000) Climate changes leads to unstable agriculture. *Trends Plant Sci* 5(9):367.
- Kempe K, Rubtsova M, Riewe D, Gils M (2013) The production of male-sterile wheat plants through split barnase expression is promoted by the insertion of introns and flexible peptide linkers. *Transgenic Res* 22(6):1089–1105.
- Tsuchiya T, Toriyama K, Yoshikawa M, Ejiri S, Hinata K (1995) Tapetum-specific expression of the gene for an endo-beta-1,3-glucanase causes male sterility in transgenic tobacco. *Plant Cell Physiol* 36(3):487–494.
- Smith MC, Thorpe HM (2002) Diversity in the serine recombinases. *Mol Microbiol* 44(2):299–307.
- Thorpe HM, Smith MC (1998) In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *Proc Natl Acad Sci USA* 95(10):5505–5510.
- Kempe K, et al. (2010) Transgene excision from wheat chromosomes by phage phiC31 integrase. *Plant Mol Biol* 72(6):673–687.
- Gleba Y, Marillonnet S, Klimyuk V (2004) Design of safe and biologically contained transgenic plants: Tools and technologies for controlled transgene flow and expression. *Biotechnol Genet Eng Rev* 21:325–367.
- Kempe K, Rubtsova M, Gils M (2009) Intein-mediated protein assembly in transgenic wheat: Production of active barnase and acetolactate synthase from split genes. *Plant Biotechnol J* 7(3):283–297.
- de Vries AP (1971) Flowering biology of wheat, particularly on view of hybrid seed production—a review. *Euphytica* 20(2):152–170.
- Evans TC, Jr., Xu MQ, Pradhan S (2005) Protein splicing elements and plants: From transgene containment to protein purification. *Annu Rev Plant Biol* 56:375–392.
- Wang Y, Yau YY, Perkins-Balding D, Thomson JG (2011) Recombinase technology: Applications and possibilities. *Plant Cell Rep* 30(3):267–285.
- Maruyama K, Kato H, Araki H (1991) Mechanized production of F1 seeds in rice [*Oryza sativa*] by mixed planting. *Jpn Agric Res Q* 24(4):243–252.
- Hawkes T, et al. (2011) D-glufosinate as a male sterility agent for hybrid seed production. *Plant Biotechnol J* 9(3):301–314.
- Conner TW, Fabbri BJ, Huang J (2002) Methods for translational repression of gene expression in plants. US Patent 20,020,062,499.
- Allen E, et al. (2007) Methods for producing hybrid seed. US patent publication WO 2007047016 (Monsanto Company, Creve Coeur, MO).
- Fritz SE, Lukaszewski AJ (1989) Pollen longevity in wheat, rye and triticale. *Plant Breed* 102(1):31–34.
- Dunwell JM (2013) Transgenic cereals: Current status and future prospects. *J Cereal Sci* 59(3):419–434.
- Rubtsova M, et al. (2008) Expression of active Streptomyces phage phiC31 integrase in transgenic wheat plants. *Plant Cell Rep* 27(12):1821–1831.
- Alexander MP (1969) Differential staining of aborted and nonaborted pollen. *Stain Technol* 44(3):117–122.