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Summary

This article reviews techniques for gene identification and cloning in allohexaploid bread wheat (*Triticum aestivum* L.). Gene identification and cloning in wheat are complicated by the large size and high redundancy of the genome. Both classical mutagenesis and transposon tagging are important tools for the study of grain dormancy and plant hormone signaling in wheat. While classical mutagenesis can be used to identify wheat mutants with altered hormone sensitivity, it can be difficult to clone the corresponding genes. We review the techniques available for gene identification in wheat, and propose that transposon-based activation tagging will be an important tool for wheat genetics.

Key words: Triticum aestivum L.; pre-harvest sprouting; dormancy; mutagenesis; transposon-tagging.

Wheat Genetics

Bread wheat (*Triticum aestivum* L.) is an allohexaploid that arose from the convergence of three diploid progenitor species. The A genome originated from *Triticum urartu* (*T. monococcum*), the B genome from *Aegilops speltoides*, and the D genome from *Ae. tauschii* (Jiang and Gill, 1994; Galili et al., 2000). The haploid number of wheat is 21, with seven chromosomes from each progenitor. Wheat has one of the largest genomes at 15 966 Mbp/1C (Arumuganathan and Earle, 1991), as compared to the human genome at 3000 Mbp/1C (McLysaght et al., 2000) or *Arabidopsis* at 145 Mbp/1C (Arumuganathan and Earle, 1991). The wheat genome's large size and high redundancy make it difficult to identify and isolate genes. Wheat genes have previously been cloned by reverse genetics (i.e., *Pina-D1*; Giroux and Morris, 1997, 1998), by homology to genes from other species (i.e., *Rht-D1*; Peng et al., 1999), by enrichment for cDNAs of interest (i.e., *PKABA1*; Anderberg and Walker-Simmons, 1992), and by labor-intensive map-based cloning (Yan et al., 2003). We are using classical mutagenesis and transposon mutagenesis to identify wheat genes controlling grain dormancy and pre-harvest sprouting.

Dormancy and Pre-harvest Sprouting

Pre-harvest sprouting (PHS) is the germination of mature grain while still in the spike. This occurs under cool, moist conditions before harvest. During pre-harvest sprouting, hydrolytic enzymes are activated and endosperm constituents are broken down, resulting in lower yield and diminished quality. Flour milled from sprouted grains loses thickening power, and breads baked from these flours have decreased volume and poor crumb structure. PHS thus lowers the grade of wheat grain from bread-quality to feed-quality, resulting in a loss of profit for the farmer (reviewed by Wahl and O'Rourke, 1993). The tendency towards pre-harvest sprouting and seed dormancy have an inverse relationship. Understanding seed dormancy should therefore give insight on controlling PHS.

Dormant embryos will not germinate, even under favorable germination conditions. All ripe wheat grain is dormant and must pass through a period of after-ripening before it can germinate. The degree of dormancy and length of after-ripening required for wheat grain germination is variable and cultivar-dependent, ranging from highly dormant with a 6 mo. after-ripening requirement to slightly dormant, with an after-ripening requirement of only a few days.

White-kernel wheats are generally more susceptible to PHS than red-kernel wheats (Groos et al., 2002). The connection between testa color and PHS susceptiblity may be due to close genetic linkage between testa color genes and genes involved in seed dormancy and PHS, or due to a pleiotropic effect of the color-controlling genes in wheat (Lawson et al., 1997; Groos et al., 2002).

Many other factors may contribute to a low degree of PHS and to seed dormancy: germination inhibitors present in the grain, reduced alpha-amylase activity in the grain, reduced water absorption by the grain, and variations in grain hormone response (Roy et al., 1999; Zanetti et al., 2000). Two plant hormones have been implicated in controlling dormancy and germination. Abscisic acid (ABA) sets up dormancy as the grain matures and dries down. ABA insensitivity has been linked to PHS in wheat (Walker-Simmons, 1987). Gibberellic acid (GA) acts antagonistically on ABA signaling and

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seed dormancy, while stimulating a germination response. These two hormones act oppositely in the control of seed dormancy. ABA and GA signaling mutants are needed to identify genes in wheat involved in hormonal control of PHS and seed dormancy.

Using Mutants for Gene Identification

Mutations can be defined as heritable changes within a gene. Before the 1920s, researchers had to rely on naturally occurring, rare mutations to study gene function. Mutagens discovered since then have greatly increased the speed and power of genetic analysis by increasing the availability of altered genes for study. Mutagens employed for gene identification in plant species can be categorized as either classical or insertional.

Classical mutagens include chemical mutagenic agents such as ethylmethane sulfonate (EMS), diethyl sulfate, azide, nitrosguanidine, and nitrosurea; and physical mutagenic agents such as X-rays, γ-rays, and fast neutrons (Rédei and Koncz, 1992). Chemical mutagens typically work by alkylating the phosphate groups of nucleotides to cause a single base change (reviewed in Heslot, 1965). Physical mutagens generally work by chromosomal breakage and/or rearrangement. A large number of mutants have been generated with classical mutagens in *Arabidopsis thaliana* L. (McKelvie, 1962; Rédei, 1970; Anderson and Mulligan, 1992), as well as in other species. Treatment of seed with classical mutagens causes mutations within the cells of the embryo. The resulting M1 plants are chimeras. Thus, mutagenized grain must be advanced to the following generation (M2) before screening to avoid genetic chimeras and allow recovery of homozygous recessive mutations (refer to Table 1).

Plants derived from insertional mutagenesis must also be grown to the M2 generation before screening for a recessive phenotype. Insertional mutagenesis has rapidly gained popularity since the 1980s (Fedoroff et al., 1984; Schell, 1987; Feldmann, 1991) and uses T-DNA (transfer-DNA) and transposon-based technologies to disrupt genes. This has great advantages over classical mutagenesis as the insert causing the phenotype is of a known sequence. This typically allows for quick and easy cloning of the interrupted gene. T-DNA is a defined segment of plasmid DNA from *Agrobacterium tumefaciens* that is transferred into the plant genome upon infection (Koncz et al., 1992). These plasmids have become tools for molecular engineering in plants. Addition of selectable markers has allowed us to use T-DNA to transform genes of interest into plants, and to mutagenize the plant genome. T-DNA insertion is fairly random. Thus, T-DNA insertion may disrupt a gene and cause a phenotype. Using T-DNA insertional mutagenesis requires a separate transformation event for each line screened. As with classical mutagenesis, mutants must be screened in the second generation (T2) to detect recessive mutations (refer to Table 1). The other method of insertional mutagenesis uses transposable elements. Transposons are DNA elements that may move from one location in the genome to another, possibly causing a phenotypic change by insertional inactivation of a gene. Transposon-tagging systems have been used to identify genes in many plant species, including maize, *Arabidopsis*, tobacco, tomato, flax, rice (reviewed by Osborne and Baker, 1995), and barley (Koprek et al., 2000).

Transposon tagging systems in plants have been based on the maize *Mutator* (*Mu*), En/Spm, and Ac/Ds transposons (Bennetzen, 1996; Enoki et al., 1999; Parinov et al., 1999; Weil and Kunze, 2000). Ac transposons have the fewest homologs in *Triticeae* (Zale and Steber, 2002). Therefore, this review will focus on the Ac/Ds two-component system for use in wheat gene identification. The Ac or *Activator* element encodes the transposase required for transposition. Ac can be 'wings clipped' by removing the 5′ and/or 3′ inverted repeats (IRs) required *in cis* for transposition; making it unable to move from its original chromosomal position. The Ds (*Dissociator*) element has all of the *cis*-sequences required for transposition, but requires the Ac transposase *in trans* to jump. In constructs for transposon tagging, the Ds element is inserted into the 5′-UTR of a selectable marker. The marker is only expressed once the Ds element transposes out, allowing selection of plants containing transposition events. To induce transposition for generating mutants, Ac- and Ds-carrying lines are crossed. The Ac-expressed transposase induces Ds to transpose in the F1 generation. Transposition events can be identified in the F2; however, homozygous recessive mutants can only be identified in the F3 generation (Fig. 1).

Progress in Developing Mutation Systems in Wheat

Mutation breeding has been used in wheat since 1951 (reviewed in Konzak, 1987). Mutation breeding in wheat is of great historical significance, as mutated *Rht-D1* and *Rht-B1* genes produced dwarf wheat varieties that started the 'green revolution' and increased production of wheat worldwide (Allan, 1986). More recently, wheat mutant screens have been for resistance to powdery mildew (Kinane and Jones, 2001), leaf rust and stem rust (Williams et al., 1992; Friebe et al., 1994; Kerber and Aung, 1995), and yellow and brown rust (Boyd et al., 2002). Success in previous mutant screens in wheat encouraged us to screen mutagenized lines for altered ABA sensitivity in an effort to understand pre-harvest sprouting.

TABLE 1

COMPARISON OF PLANT MUTAGENESIS METHODS

Mutagen	Lesion	Type of mutation	Generation screened
X-ray, fast neutron, γ-ray	Chromosome breakage	Deletion and chromosome rearrangement	M2
EMS, diethyl sulfonate, azide	Single base change	Missense and nonsense	M2
T-DNA	Insertion	Disruption	T2
Transposon	Insertion	Disruption	T3
Activation tagging via T-DNA or transposon	Insertion	Disruption or overexpression of downstream gene	T2 or T3

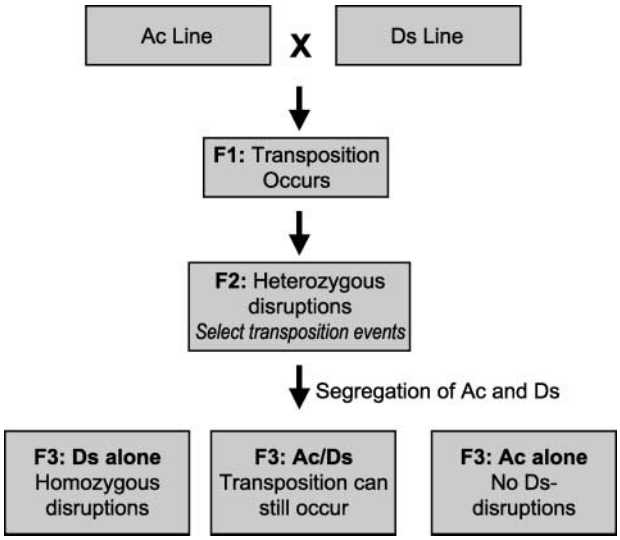

FIG. 1. Using transposons to generate mutants in plants.

Classical mutagenesis. Screens for altered ABA sensitivity in germination have yielded many different types of mutations in *Arabidopsis thaliana*. ABA-insensitive germination screens yielded several mutants with various phenotypes. Mutants such as *Ab*scisic acid-*i*nsensitive *1-1* (*abi1-1*) and *abi2-1* produce non-dormant seeds and have wilty vegetative phenotypes (reviewed in Finkelstein and Rock, 2002). On the other hand, other *Arabidopsis* mutants selected for the same ABA-insensitive germination phenotype (*abi3*, *abi4*, and *abi5*) have non-dormant seeds, but little to no vegetative phenotype change. We have performed an ABA-insensitive germination screen in wheat, based on this previous work in *Arabidopsis*. From screening 7320 chemically mutagenized (EMS) M2 wheat grains, we have obtained 11 independent ABA-insensitive lines (Strader and Steber, unpublished). Some mutant lines from this screen demonstrate only the ABA-insensitive germination phenotype, while others demonstrate both germination and wilty vegetation phenotypes.

The complementary screen for increased ABA response has also been done in *Arabidopsis*. From these screens, seeds displaying an *e*nhanced *r*esponse to *A*BA (*era*) during germination were isolated. The mutants *era1* and *era3*, as well as *abh1* (*ABA-h*ypersensitive), have increased seed dormancy. Both *era1* and *abh1* also have increased drought tolerance (reviewed in Finkelstein and Rock, 2002). A previous study isolated a reduced grain dormancy mutant in wheat, but the identity of this single, dominant mutation remains unknown (Kawakami et al., 1997). We performed an ABA-hypersensitive germination screen in wheat, based on this previous work. From screening 22 520 fast-neutron mutagenized M2 wheat grains, we obtained 39 independent lines with this phenotype (Strader and Steber, unpublished).

Both of these screens have limitations in a polyploid system. Multiple copies of the same gene represented on different genomes make it difficult to isolate recessive, loss-of-function mutants with the desired phenotype. However, the fact that recessive mutants can be recovered (Kerber, 1991) causes one to wonder if intricate gene regulation of the multiple copy genes is occurring, and whether the plants will activate expression of homeologous genes on the other genomes to recover from the loss of function of one copy. Following the phenotypes of isolated recessive mutants through several generations should give an indication as to whether this type of control is occurring. A solution to this limitation is to find a way to create stable, dominant mutations that can be made independent of genome copy number. An activation tagging system should allow one to overcome polyploid problems in mutant searches.

Insertional mutagenesis and TAT system development. Activation tagging was first performed in plants by Hayashi et al. (1992) using a T-DNA vector containing four copies of the CaMV 35S promoter in tandem near the right border of the T-DNA. This caused overexpression of sequence downstream of the T-DNA, allowing them to identify dominant tobacco mutants for auxin-independent growth. Since then, activation tagging via T-DNA insertion has been used to develop a number of stable dominant and semi-dominant tagged mutants in *Arabidopsis* (Weigel et al., 2000). Activation tagging has also been performed in *Arabidopsis* using the Ac/Ds transposon system containing a single copy of the CaMV 35S promoter at the 3′-IR (Long et al., 1997). This was the beginning of transposon-based activation tagging (TAT).

We feel that transposons are the best method for accomplishing activation tagging in wheat. Activation tagging enriches for dominant mutations, because genes downstream of the insertion carrying the 35S promoter are overexpressed, producing the phenotypic change. This is useful in a species with a large genome, where gene redundancy is able to mask most deletion mutations. Transformation in wheat can be laborious and time-consuming, taking several months to a full year to regenerate plants from tissue culture after the transformation event. Activation tagging using the T-DNA systems requires a separate transformation event for each gene tagged, whereas generating TAT mutants by cross-pollinating wheat Ac- and Ds-carrying lines is a more expedient means of producing activation-tagged mutants. We are currently in the process of creating an Ac/Ds TAT system in wheat. Preliminary data suggest that the Ac/Ds system is operational in wheat (Zale and Steber, unpublished data). We believe both traditional mutant screens and activation tagging can be successful in polyploids. Although creating classical mutants is more quickly achieved than creating insertional mutants, cloning of activation tagged genes should be far easier to accomplish in a polyploid system.

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