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PERENNIAL WHEAT BREEDING:
CURRENT GERMPLASM AND
A WAY FORWARD FOR BREEDING AND
GLOBAL COOPERATION

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ABSTRACT

The Australian perennial wheat team has collected and assessed a wide range of global germplasm derived from wheat x perennial-relative crosses (Hayes *et al.* 2012 and unpublished). Some lines were able to perenniate in one or both field environments where they were grown and a few

were able to regrow through four seasons. Without exception the only wheat-derived lines that could perenniate contained seven or more pairs of chromosomes from the perennial parent. The donor perennial parents included *Thinopyrum ponticum* (2n = 70, decaploid), *Th. intermedium* (2n = 42, hexaploid) and *Th. elongatum* (2n = 14, diploid). This has led us to conclude that the best near-term prospect for a perennial wheat-like grain crop is a full or partial amphiploid, containing the full set of tetraploid (AABB) or hexaploid (AABBDD) wheat chromosomes plus one genome (XX) from the donor. When the perennial donor is a polyploidy, the extra genome is usually a synthetic genome, consisting of a mixture of chromosomes from the polyploid parental genomes. This creates a significant difficulty in that each time a primary partial amphiploid is produced, the synthetic genome may consist of a different mix of donor chromosomes, and therefore interbreeding of these primary partial amphiploids may result in poor fertility and loss of the perennial donor chromosomes. That, in turn, would be problematic in trying to establish a breeding program. Consequently we would advocate a breeding program based on a diploid perennial donor, such as *Th. elongatum* (EE). This is analogous to triticale breeding. The amphiploids produced could be AABBEE (analogous to hexaploid triticale, AABBRR) or AABBDDEE (analogous to octoploid triticale, AABBDDRR). We would further advocate a multinational effort to produce many primary amphiploids using locally adapted wheat parents and diverse accessions of *Th. elongatum*; the primary amphiploids would be shared among participating groups, and intercrossed to permit subsequent selection of robust, productive, locally adapted perennials. The analogy with triticale is instructive also in suggesting that many generations of selection may be required before full fertility, high yielding types can be recovered.

Keywords: amphiploidy, genomic changes, perennial wheat, rye, *Thinopyrum*, triticale

INTRODUCTION

This paper explores the question of the preferred breeding route to a successful wheat-like and wheat-derived perennial cereal. Other papers in the Workshop will explore related aspects such as the physiology and field performance of available perennial wheat germplasm and how perennial wheat might usefully fit into a mixed farming situation. Some of that information is assumed as a starting point for this consideration of the best genetic configuration for a perennial wheat and how to instigate a breeding program to move us from basic proof of principle to genotypes that can be successfully and usefully deployed into real farming systems.

LIKELIHOOD OF A PERENNIAL TRUE WHEAT

Our studies of the available germplasm have established the essential feasibility of the concept of a perennial wheat (Hayes *et al.* 2012 and unpublished). Lines were found that could regrow after grain harvest for up to four seasons; simulated grazing of several lines showed an ability to produce both valuable forage and grain; and in addition we could demonstrate their ability to accumulate much greater root biomass after the first season as an indicator of the environmental benefit that could accrue (Larkin *et al.* 2014). The grain quality of these lines even had potential for bread making, and we along with others have also demonstrated the disease resistances that had been contributed from the donors of perenniality (Cox *et al.* 2002; 2005; Murphy *et al.* 2009; Hayes *et al.* 2012). Despite this promise, few of the lines were developed from adapted wheat species and their grain yields were generally low and declining in subsequent years. The available germplasm does not meet the standard required for robustness of the perennial habit or the consistency of grain yield from one season to the next. What we concluded for the Australian environment, Murphy *et al.*(2010), Cox *et al.*(2010) and Jaikumar *et al.*(2012) concluded for North America, namely that better adapted and more productive germplasm is required. For decades perennial wheat breeding attempts in the United States and the former USSR held some hope that it might be possible to introgress the controlling genes for the perennial habit into the wheat genomes through recombination or chromosomal translocation. Indeed Lammer *et al.* (2004) gave impetus to this hope when they showed some capacity to regrow in the wheat cv. Chinese Spring, carrying an extra pair of 4E chromosomes from *Thinopyrum elongatum*, a perennial *Triticeae* species. However, the ability of this chromosome addition line to regrow and set seed a second time was not as strong as the perennial amphiploid progenitor, which itself was not very robust. Perenniality appears to be a polygenic trait (Zhao *et al.* 2012), and it will not be readily conferred by simple introgression from a perennial to an annual species. Indeed the likely genetic complexity of the perennial habit suggests the possibility that it might be easier to transfer domestication traits to an existing perennial *Triticeae* species. These traits would include non-shattering heads, free-threshing grain, more determinate flowering and larger seed size. The genetic controls for some domestication traits are relatively simple (Faris *et al.* 2003; Sang *et al.* 2009; Gegas *et al.* 2010; Takahashi and Shimamoto, 2011; Peng *et al.* 2011). Others such as threshability may be multigenic (Peleg *et al.* 2011).

PERENNIAL GRAIN BY DOMESTICATION

In Australia some herbaceous native legumes have been investigated as having potential as perennial grain crops through a process of domestication (Bell *et al.* 2012). Attempts have been

made to commercialize grasses such as *Microlaena stipodies* and *Distichlis palmeri* as perennial grain crops (O'Neill, 2007; Kasema *et al.* 2010; Pearlstein *et al.* 2012). However, this has met with limited success. For the Australian native rice-related perennial grass *M. stipoides,* the major missing traits included synchronous maturity and resistance to shattering (Davies *et al.* 2005). The seeds are structurally similar to rice and some ecotypes have seeds almost as large as domesticated rice. Malory *et al.* (2011) characterized 18 genes from *Microlaena* which are homologues of rice genes known to be important in domestication. If successful, domestication of wild perennial grains will produce crops, which are only likely to have value for niche markets in the medium term. Lower flour yields and higher bran and fibre content per kernel are often an impediment to acceptance in standard milling markets for new domesticated grains (Bell *et al.* 2010). We will hear more from others at this meeting on the prospects for domesticating perennial species.

The Land Institute is also making good progress in direct domestication of a number of perennial species including the wheat relative *Thinopyrum intermedium* (kernza) (DeHaan *et al.* 2005; Van Tassel *et al.* 2010; Culman *et al.* 2013). What might be needed to advance success more rapidly down this domestication option is to utilize the expanding understanding of domestication genes in a more targeted way. Transgenic technology offers a powerful way to test the utility of specific genes in achieving domestication traits. Thereafter, if the course to commercial release is deemed too difficult for the transgenic plants, the same traits might be pursued through targeted mutations using technology such as TILLING or one of the emerging methods for site-directed genome modifications (Curtin *et al.* 2012). Genome editing technology employs sequence-specific nucleases to generate site-specific double-stranded Deoxyribonucleic Acid (DNA) breaks. Nuclease systems in development include: zinc finger nucleases; transcription activator-like effector nucleases (TALENs); and LAGLIDADG homing endonucleases (Curtin *et al.* 2012). The site-specific double strand DNA breaks are followed by informed and designed mutations, sequence insertions or replacements. Rapidly evolving genomic and molecular genetic technology may make rapid domestication of existing well-adapted perennials feasible, but not without substantial investment in the various species of interest to this Workshop and for the goal of a perennial grain crop.

PERENNIAL CEREAL GRAIN THROUGH HYBRIDISATION AND ALLOPOLYPLOID FORMATION

Hybridisation between annual grain crops and perennial relatives offers an avenue to combine the traits of perenniality and grain quality in a new crop species. We believe this would reduce the time of development, compared with domestication, and offer a product that might directly replace annual grains in the market place. There have been attempts to achieve this historically

in Russia and the United States (Tsitsin and Lubimova, 1959; Suneson *et al.* 1964). Experimental breeding lines of hybrid perennial cereals have shown higher mineral and protein content than annual cultivars (Murphy *et al.* 2009) and some have demonstrated useful flour, dough and baking properties (Hayes *et al.* 2012). Perennial grains will need to be profitable if they are to be adopted widely in agriculture (Bell *et al.* 2008; Reeling *et al.* 2012) and direct access to already established markets will be important for the acceptance of perennial grains.

The rest of this paper focuses on wide hybridisation to combine perenniality with already domesticated wheat. Our experience thus far consistently indicates that reasonable rates of post-harvest regrowth (PHR) are only observed when many chromosomes are added to wheat from the perennial donor species (Hayes *et al.* 2012 and unpublished). The germplasm examined was derived from wide crosses between wheat and perennial species such as *Th. intermedium, Th. elongatum,* and *Th. ponticum*. Figure 1 is a plot of many of the hybrid derivatives, partial amphiploids and amphiploids that we have examined in the field, showing the relationship between chromosome counts and ability to regrow after the first grain harvest. This figure is based on a similar figure in Hayes *et al.* (2012), but with some new lines and field data added. Some lines were able to perenniate in one or both field environments where they were grown and a few were able to regrow through four seasons (Table 1). The wheat-derived lines that were capable of perennial regrowth contained seven or more pairs of chromosomes from the perennial parent. In the case of the wheat x *Th. elongatum* derivatives, the exception seems to be CPI147232, which has 42 chromosomes but is likely to have substituted the E genome for one of the wheat genomes, just as in the durum wheat x *Th. elongatum* amphiploid (Figure 1A). The situation is even clearer for wheat x *Th. intermedium*, wheat x *Th. ponticum* and wheat x unknown *Agropyron sp.* derivatives, where only 56 chromosomes lines show significant perenniality (Figure 1B). Individual *Triticeae* genomes have seven pairs of chromosomes, and therefore, the chromosome constitutions of stable allopolyploids, both naturally occurring and synthetic amphiploids, stabilise at multiples of 14. Many fertile and stable wheat derived amphiploids have been formed at the octoploid level (2n = 56; Mujeeb-Kazi and Hettel, 1995; Jauhar, 1995; Sepsi *et al.* 2008), and it seems this is a benchmark of stability as well as a general requirement for retaining perenniality. Figure 1 also illustrates that although the wheat hybrid derivatives do not regrow with the same consistency as perennial benchmarks such as *Th. intermedium* or *S. montanum*, some do well.

TABLE 1. SUCCESSIVE GRAIN YIELDS OF HYBRID WHEAT DERIVATIVES FROM WHEAT X *TH. ELONGATUM* OR WHEAT X *TH. INTERMEDIUM*, AND THE PERENNIAL GRASS *TH. PONTICUM*, GROWN IN AUSTRALIA

The weights are means of three replicate 1 m rows. Decline in yield, of the hybrids, over time was due to plant mortality within populations.

	GRAIN wt (g) 2008	GRAIN wt (g) 2009	GRAIN wt (g) 2010	GRAIN wt (g) 2011
CPI 147235a	73.5	25.9	0.0	0.0
CPI 147236a	81.7	45.9	11.2	0.0
CPI 147236b	65.9	8.9	0.0	0.0
CPI 147251b	70.9	9.4	0.5	0.4
CPI 147257b	7.8	12.6	4.7	0.7
CPI 147258a	4.7	0.3	0.3	0.0
CPI 147286a	36.3	0.4	0.0	0.0
Th. ponticum	0.0	9.4	207.7	268.8
lsd	45.7			

FIGURE 1. CHROMOSOME COUNTS (2) AND POST-HARVEST REGROWTH (PHR) SCORE IN THE FIELD FOR A RANGE OF CONTROL PERENNIAL SPECIES AND WHEAT HYBRID DERIVATIVES

The bars show the range where variable chromosome counts were observed. Some of the data plotted are from Hayes *et al.* (2012).
A. shows mainly wheat x *Th. elongatum* derivatives.
B. shows mainly wheat x *Th. intermedium* derivatives.

A. *T. AESTIVUM* X *TH. ELONGATUM* DERIVATIVES AND *S. MONTANUM*

B. *T. AESTIVUM* X *TH. INTERMEDIUM* DERIVATIVES AND CONTROLS
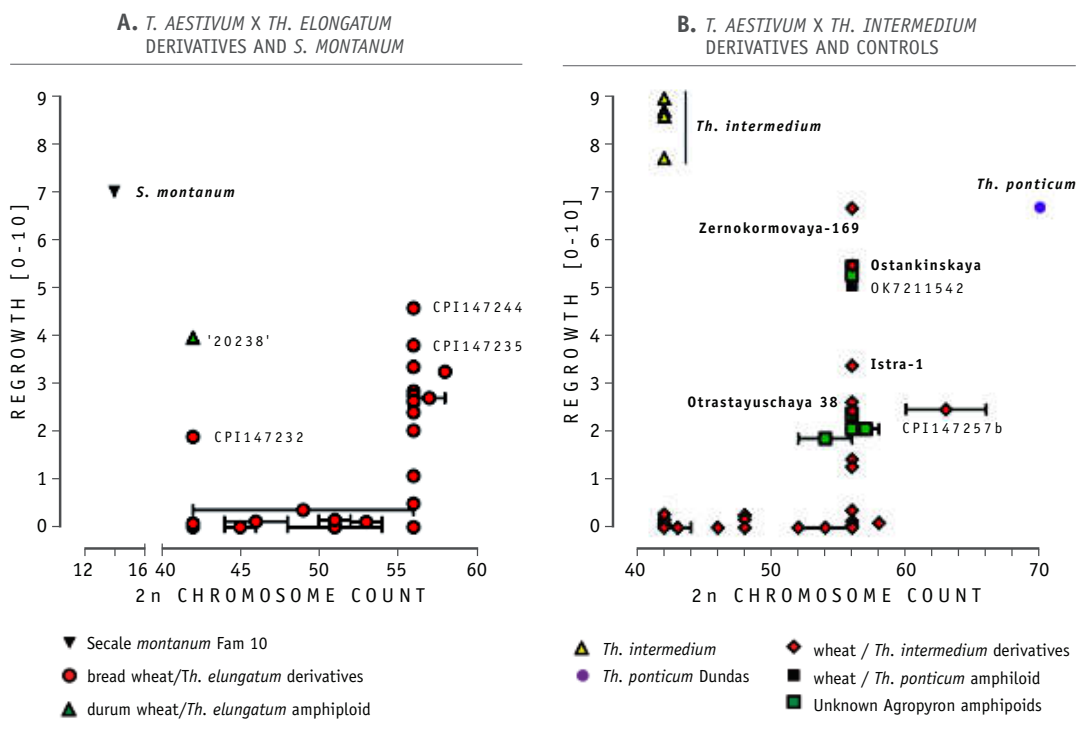
Perennial amphiploids or partial amphipoids have been produced between wheat and *Th. ponticum* (2n = 70, decaploid) (Berezhnoi, 1987; Zhang *et al.* 1996; Chen *et al.* 1998), *Leymus arenarius* (2n = 56, octoploid) (Anamthawat-Jonsson, 1995), *Th. intermedium* (2n = 42, hexaploid) (Tsitsin and Lubimova, 1959; Cauderon, 1966; Sun, 1981; Jones *et al.* 1999; Cox *et al.* 2002) and *Th. elongatum* (2n = 14, diploid) (Jenkins and Mochizuki, 1957; Rommel and Jenkins, 1959; Cai *et al.* 2001; Murphy *et al.* 2007; Mujeeb-Kazi *et al.* 2008). Taken together, this experience suggests that the best near-term prospect for a perennial wheat-like grain crop is a full or partial amphiploid, containing the full set of tetraploid (AABB) or hexaploid (AABBDD) wheat chromosomes plus one genome equivalent (XX) from the donor. If the perennial donor is a polyploidy, the extra genome in the allopolyploid (partial amphiploid) is usually a synthetic genome. In this context, "synthetic genome" means one consisting of a mixture of chromosomes from the multiple perennial parent genomes, but where each of the seven homologous chromosome groups are represented in the synthetic genome. If each of the homologous groups are not represented the synthetic genome is unstable. So, while a partial amphiploid with a synthetic genome may be stable, a significant difficulty results for any ongoing breeding program. Each time a primary partial amphiploid is produced, the synthetic genome may consist of a different mix of donor chromosomes (Jones *et al.* 1999; Han *et al.* 2004; Liu *et al.* 2005), and therefore interbreeding of primary partial amphiploids often will result in a loss of the donor chromosomes, genetic instability and consequent likely loss of the perennial trait. Banks *et al.* (1993) amply demonstrated this problem through experiments in which a set of independent primary wheat-*Th. intermedium* partial amphiploids (2n = 56) were inter-crossed. We would argue that the ability to inter-breed from independent primary amphiploids is essential to enable a practical rate of breeding progress with hybrid perennial wheats. Robust perennials might be achievable with partial amphiploids using a polyploidy perennial donor; some of the best performing lines in our experiments have derived from polyploid donors such as wheat x *Th. ponticum* (10x) derivatives or wheat x *Th. intermedium* (6x) (Figure1B). However, substantial progress will be made best, if ongoing breeding programs can be established through developing genetically diverse primary amphiploids, inter-crossing them to generate large scale genetic segregation on which to impose heavy selection.

Consequently, although perennial allopolyploid *Triticeae* might make good donors for primary partial amphiploids, we believe the best prospects for a productive breeding program in the medium term should focus on a diploid perennial donor such as *Th. elongatum* (Host) D. R. Dewey (EE, 2n = 2x = 14, synonyms *Lophopyrum elongatum, Elytrigia elongata, Elymus elongatus, Agropyron elongatum*). The Washington State University (WSU) program used this donor species introduced as the Chinese Spring wheat x *Th. elongatum* amphiploid they called AgCs (Jenkins, 1957; Cai *et al.* 2001). It is helpful to consider the analogy with the breeding of the human-made cereal triticale. If a tetraploid wheat is used, the amphiploid would be AABBEE (analogous to hexaploid triticale, AABBRR); if a hexaploid wheat is used, the amphiploid would be AABBDDEE

(analogous to octoploid triticale, AABBDDRR). Lelley (2006) gives a useful summary of the history and breeding of triticale. The first fertile triticale, an octoploid, was produced by Rimpau in 1888. It was only in the 1930s, that induced chromosome doubling techniques using colchicine enabled routine production of fertile primary triticales. Research switched to hexaploid triticales in the 1950s, and progress was made by extracting secondary hexaploids from crosses between hexaploid and octoploid triticales. The International Maize and Wheat Improvement Center (CIMMYT) achieved another step forward by producing hexaploid cv. Armadillo carrying a 2D (2R) chromosomal substitution, though subsequent developments have shown that the full potential of triticale is best served with a full rye genome and without substitutions. Decades of breeding and selection have been required to produce high yielding triticale cultivars. There is evidence that during this time genomic sequence modifications were important (Ma and Gustafson, 2008; Tang *et al.* 2008; Ozkan and Feldman, 2009; Feldman and Levy, 2012). The observed changes were mainly losses of Amplified fragment length polymorphism (AFLP) and Restriction fragment length polymorphism (RFLP) bands, predominantly from the rye genome and largely involving repetitive DNA sequences. A positive relationship was found between increased chromosome bivalent pairing in meiosis, improved fertility and elimination of low-copy non-coding DNA sequences (Ozkan and Feldman, 2009). These chromosomal rearrangements may have facilitated the wheat and rye genomes coordinating the timing of their division processes during meiotic division. Both genetic and epigenetic changes have been demonstrated to occur in newly synthesized allotetraploid wheat lines with diverse genome compositions (Qi *et al.* 2010). Some of the changes seemed to be required, which they called *directed*, while others were highly variable, which they called *stochastic*. Feldman and Levy (2012) prefer the word *revolutionary* to describe rapid changes required in new allopolyploids. The changes observed included non-random loss of non-coding, low- and high-copy sequences, resulting in a DNA loss of 2-10 percent compared with the sum of the parental DNA contents. In the case of triticale, the loss was about 9 percent in octoploid triticale and 28-30 percent in hexaploid triticale, compared with the wheat and rye parental DNA. Intergenomic invasion by sequences such as transposons, and reduction in repeats of Ribosomal ribonucleic acid (rRNA) genes are also noted as revolutionary genetic changes occurring rapidly after allopolyploidisation. Epigenetic (DNA methylation) changes were also observed. More recently Hu *et al.* (2012) looked specifically at newly formed wheat x *Th. elongatum* amphiploids using genome specific molecular markers and found chromosome rearrangements and sequence duplications occurring. Interestingly, the whole genome shot-gun sequencing of wheat has revealed the apparent dynamism of hexaploid wheat genomes, in which there has been significant loss of members of multigene families during polyploidization and domestication (Brenchley *et al.* 2012).

Most of the wheat x *Th. elongatum* amphiploids we have examined have been from WSU and are octoploid (2n = 56, presumably AABBDDEE). However, one of the perennial WSU lines, CPI147232, is hexaploid (2n = 42). Its genomic composition needs to be confirmed.

Another hexaploid amphiploid is derived from cv. Stewart (*T. turgidum*) x *Th. elongatum* line (AABBEE) (Jenkins and Mochizuki, 1957). This hexaploid is too tall but should be crossable to CPI147232, provided the latter is also AABBEE, in which case the progeny should segregate to form populations at the hexaploid level useful for selections. Almouslem and Amleh (1999) also report a durum wheat x *Th. elongatum* hybrid. The greater genomic proportion of the perennial genome to annual genomes may confer a more dominant perennial habit; however this remains to be tested.

More recently we have obtained new wheat x *Th. elongatum* amphiploids at the octoploid level from CIMMYT, in particular two lines using wheat cv. Goshawk (Mujeeb-Kazi *et al.* 2008). These had good semi-dwarf stature, strong straw and reasonable PHR in the greenhouse. However, fertility was reduced and the heads were very susceptible to shattering (Larkin, unpublished). Shattering was not a problem with the perennial wheat *Th. elongatum* amphiploids from WSU. Crosses between the two types have been successful. And hopefully will allow the recovery of progeny with non-shattering heads, but with semi-dwarf stature and improved fertility. *Th. elongatum* accessions are available from dry and hot locations such as Israel, North Africa and Mediterranean France. Although this diploid species is not endemic in Australia, some accessions may exhibit the type of summer survival and persistent perenniality required. These should be exploited to make new primary amphiploids with adapted annual wheats.

Th. elongatum is not the only perennial diploid that might serve as the donor of the perennial habit to wheat. It is noteworthy that most species of the *Triticeae* are perennial (Barkworth *et al.* 2009) with about ten basic genome types represented, including *Australopyrum retrofractum* (W), *Agropyron cristatum* (P), *Thinopyrum bessarabicum* (Eb), *Dasypyrum villosum* (V), *Psathyrostachys hushanica* (Ns), *Pseudoroegneria spicata* (St); and wheat can be hybridised to some of these species. So there should be many other possibilities for generating perennial amphiploids. The diploid *Australopyrum* species (2n = 2x = 14, WW) might appear to be an attractive donor of perenniality in an Australian context. However, there are no reports of hybrids with wheat. Furthermore, *Australopyrum* species lack the broader adaptation to various climatic regions of southern Australia associated with the other native perennial *Triticeae* species, *Elymus scaber*.

Some attention has been given to producing a perennial triticale derived from perennial rye, *Secale montanum,* instead of cereal rye to combine with wheat. Schlegel (1980) produced F1 hybrids and amphiploids from wheat x *S. montanum* crosses; the amphiploids at meiosis had an average of 26.55 bivalents compared with 27.30 in an established octoploid triticale, suggesting a reasonable genetic stability early in the breeding cycle. There was a correlation between the amount of telomeric rye heterochromatin and the frequency of univalents in meiosis, suggesting the heterochromatin difference between the wheat and rye chromosomes was an impediment to full fertility. Delayed DNA replication at the heterochromatic telomeres may be a problem for coordinating the timing of wheat and rye chromosome division processes during meiotic division. The reduced amount of telomeric heterochromatin in *S. montanum* relative to *S. cereale*

is postulated to explain why wheat *S. montanum* amphiploids were more regular in pairing than primary wheat *S. cereale* amphiploids (Thomas and Kaltsikes, 1974; Schlegel, 1980). Established fully fertile triticales have lost much of the telomeric heterochromatin on the rye chromosomes. This is another example of a genetic change required to enable newly formed allopolyploids to be fully fertile.

There has been some success in breeding perennial rye itself as a grain crop through intercrossing *S.cereale* and *S. montanum* and the release of tetraploid cvs. Permontra and Sopertra, diploid cv. Benmonta in Germany (Reimann-Philipp, 1995), and diploid cvs. Perenne and Kriszta in Hungary (Kotvics *et al.* 2001; Füle *et al.* 2005; Sipos and Halasz, 2007). In Canada, cv ACE-1 was developed by selection from German germplasm (Acharya *et al.* 2004). Initial attempts had difficulties with fertility and retention of perenniality. Success came only after decades of selection. In Australia, a perennial rye cv. Black Mountain was bred from a *S. cereale x S. montanum* cross with two backcrosses to the perennial parent; selection for this release was based mainly on perenniality and forage yield, rather than grain yield. Following cell culture and six generations of selection for fertility, non-shattering heads and grain yield, a higher grain yield perennial selection was recovered called Family 10 (Oram, 1996; personal communication, 2010).

CONCLUSIONS

With the increasing power and reach of genomic analyses, greater genetic insight will come eventually into the genes controlling the perennial habit. Transcriptomic comparisons between annual and perennial *Brachypodium* species, being arguably the closest model species to wheat, might prove particularly informative. Other potentially informative comparisons include annual and perennial rice (*Oryza rufipogon*) (Zhao *et al.* 2012), *Sorghum bicolor* and *S. halepense*, *Hordeum vulgare* and *H. bulbosum, Panicum miliaceum* and *P. turgidum*. Eventually such studies could lead to the ability to engineer perenniality into wheat through transgenics or genome editing. In the meantime we would contend that the best near term prospect of a productive breeding program for a perennial wheat-derived cereal will involve the following steps:

1. The generation of many primary amphiploids between wheat and a perennial diploid such as *Th. elongatum*. Importantly, this should involve a diversity of *Th. elongatum* accessions and a diversity of annual wheat cultivars adapted to various target zones globally.
2. Intercrossing primary amphiploids and advancing segregating populations with selection.
3. Early generation selection at F2 to F4 would emphasise traits such as semi-dwarf plant height, non-shattering heads, stable amphiploid chromosome count, large seed size, good self-fertility, and regrowth in pots.
4. Later generation selections would emphasise traits such as maturity, robust post-harvest growth, grain yield, forage yield, stability of grain yield across seasons, and disease resistance.

In addition to a number of rounds of such breeding and trait selection cycles, progress might be further accelerated through specific attention and selection for the *revolutionary* genetic changes so frequently observed in newly forming allopolyploids (Feldman and Levy, 2012), that signal the accommodation between genomes and the rise in fertility and stability. While triticale took over 40 years to begin to deliver on its potential, the time required to deliver a successful perennial wheat might be greatly compressed through a coordinated international effort and application of our greater understanding of the genomic changes required before a newly formed allopolyploid becomes stable and productive. International cooperation could be encouraged through the formation of a *community-of-practice* with mutually agreed *open-source* style terms of engagement. We already have the beginnings of such a community. High priority should be given to collecting diverse accessions of diploid *Th. elongatum*, crossing to wheat and forming amphiploids, sharing diverse primary amphiploids, intercrossing to form secondary amphiploids, and coordinated multi-environment testing of derived secondary amphiploid populations.

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