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CURRENT EFFORTS TO DEVELOP PERENNIAL WHEAT AND DOMESTICATE *THINOPYRUM INTERMEDIUM* AS A PERENNIAL GRAIN

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ABSTRACT

We are developing a new perennial grain by domesticating the perennial grass *Thinopyrum intermedium* (intermediate wheatgrass). In 1983, intermediate wheatgrass was selected for domestication by the Rodale Institute (Kutztown, Penn., USA). Nearly 100 species of perennial grasses were evaluated for promise as a perennial grain before choosing intermediate wheatgrass to domesticate. The Rodale Institute performed two cycles of selection, beginning in 1988. Using selections made by Rodale, breeding work began at The Land Institute (Salina, Kan., USA) in 2002.

Selection has been for yield per head, increased seed mass, free threshing ability, reduced height, and early maturity. Two cycles of selection at The Land Institute have increased seed yield by about 77 percent and seed mass by about 23 percent, when grown in a solid stand. Selected materials have been found to possess a higher harvest index and reduced plant spread.

Molecular tools are being developed for intermediate wheatgrass. A combination of expressed sequence tag (EST), SSRs and AFLP markers will be used to genotype an experimental mapping population comprises 268 full-sib progeny derived from a reciprocal cross of two experimental genotypes. Genotyping by sequencing is also being used to identify ~3 000 high quality single-nucleotide polymorphisms (SNPs) in a population derived from one selfed individual. Phenotype data has been collected and will be used to identify QTL associated with SNPs. Using gene cloning, protein separation and identification, and sequence alignments; we were able to identify five HMW-GS genes and their allelic variants in intermediate wheatgrass plants.

Since 2001, we have been working to develop perennial wheat by crossing wheat (*Triticum* spp.) with perennial *Thinopyrum* species. We have obtained a few stable lines with one set (12-14) *Thinopyrum* chromosomes and 42 wheat chromosomes. These have better agronomic performance in Kansas than other materials, but lack perenniality. Crosses between winter durum wheat and *Thinopyrum intermedium* have been recently developed, and they are promising in terms of perenniality, seed weight, winter hardiness, and vigor. To study the impact of annual/ perennial genome dosage on perenniality and agronomic performance, we have crossed diploid and tetraploid wheat lines with tetraploid and hexaploid *Thinopyrum* species. All F_1 plants are perennial, and many have been doubled with colchicine. Wheat chromosome-specific markers have been used to characterize 94 plants that were perennial in the field. Plants with more wheat chromosomes eliminated tended to be more perennial, but specific chromosomes were not associated with perenniality or annuality.

INTRODUCTION

Two major approaches are being used to develop perennial small grains: wide hybridization and domestication. The two approaches present unique strengths and challenges. Wide hybridization involves crossing an annual grain such as wheat with related perennial species. Wide crosses will in theory make available genes controlling traits such as yield, seed size, free threshing ability, and quality, which have been accumulated in current grain crops. With wheat the challenge has been to obtain cytogenetic stability in wide hybrids while preserving perenniality and domestication traits. Direct domestication of wild perennials has the potential benefit of working with populations that are vigorous perennials. However, the necessary genetic variation for domestication may be lacking in perennial species, or substantial time may be required for selection to achieve adequate seed size, yield, or other domestic traits.

The first sustained effort to directly domesticate a perennial grass for grain, of which we are aware, was initiated by researchers at The Rodale Institute in Pennsylvania, United States of America (Wagoner, 1990). The researchers leading this domestication effort cite Jackson (1980) as the inspiration for developing perennial grasses for grain. In 1983, researchers began to evaluate nearly 100 perennial grasses to determine their suitability for grain production. Based on numerous criteria, intermediate wheatgrass (*Thinopyrum intermedium*) was selected as a perennial grain candidate. Beginning in 1988, two cycles of selection for improved fertility, seed size, and other traits were performed in Pennsylvania.

Selection for grain production in intermediate wheatgrass was continued by workers at The Land Institute in Kansas, United States of America, beginning in 2003 (Cox *et al.* 2010). Four cycles of selection using an index based primarily on seed yield per head, seed mass, and free threshing ability have been performed at The Land Institute. Improved populations resulting from this selection program are currently being evaluated and further selected by collaborators in diverse environments.

We began a perennial wheat (*Agrotriticum*) breeding program in 2001. Perennial wheat has potential in reducing soil erosion and promoting the sustainability of agriculture (Cox *et al.* 2002; Cox *et al.* 2006). Through perennial wheat, or wheat-*Thinopyrum* hybrids, more than 18 agronomically important traits other than perenniality have been transferred from *Thinopyrum* species into wheat (Chen *et al.* 2005). At the beginning of our breeding program, we introduced perennial wheat lines from other institutions. All of them died at a time point after grain harvest during the hot summer of Kansas (Cox *et al.* 2006). Therefore, we have sought fresh approaches to developing truly perennial wheat. Among these new approaches are management techniques. Because we have yet to identify agronomic strategies to induce perenniality in Kansas, here we will focus on genetic approaches.

RECENT PROGRESS

Breeding Perennial Wheat

In our search for an approach to perennial wheat with good grain yield and perenniality, we have crossed wheat with *Th. ponticum* (10x = 70), *Th. intermedium* (6x = 42), and *Th. elongatum* (2x = 14). We have also attempted crosses with other *Thinopyrum* species. Crosses involving *Th. junceiforme* (4x = 28), *Th. bessarabicum* (2x = 14), and a *Th. bessarabicum*-*Th. elongatum* hybrid (4x = 28) have produced hybrid F_1 plants successfully. The crosses with *Th. junceum* (6x = 42) and *Th. scerpium* (4x = 28) could form well-developed embryos. But the plantlets died shortly after germination on nutrient medium. One *Thinopyrum* accession (6x = 42; PI531731) is unique in that it is strictly self-pollinated. We have attempted to cross 6x and 4x wheat with this accession. However, no hybrid embryos have been obtained as the caryopses aborted at early stage. We have successfully obtained F_1 hybrids between *Thinopyrum* species and both rye and triticale. So far, no crosses involving rye have resulted in lines with perenniality and seed fertility.

In recent years, we have created a couple of genetically stable lines which were derived from the crosses between common wheat (*T. aestivum*) and *Th. intermedium* wheatgrass. Similar to a great number of perennial wheat lines developed by other institutions, these lines had chromosome numbers from 54 to 56 with 12 to14 chromosomes from wheatgrass. Minor translocations involving wheatgrass chromosomes were observed on some wheat chromosomes. In our plot experiments, four breeding lines named B373, B1126#1, B1126#2 and B1321 did much better than the others. They looked similar to annual wheat cultivars, ripened early, and had excellent seed fertility and better-filled grain. Interestingly, these four lines shared a common parent, B373. They were similar to B373 in many aspects morphologically, but had improved regrowth ability. The line B373 was just a few days later in maturity than local common wheat cultivars. The wheat cultivar Jagger, a leading wheat cultivar in Kansas from the 1990s through the 2000s, was the donor of wheat chromosomes to B373. 'Jagger' might have contributed to the improved adaptability of these lines. By contrast, perennial wheat lines from the former Soviet Union and Washington State did not present similar adaptability. They flowered extremely late, produced fewer heads, and filled grain poorly.

In a root-tube experiment, we compared the three "perennial" wheat lines B373, B1126#2, and OK7211542 with wheat cultivar Jagger and a *Th. intermedium* selection C3-2627. The total biomass, root biomass, shoot biomass, and grain weight of the three perennial wheat lines are intermediate to the wheat and wheatgrass controls, respectively, except that OK7211542 had larger total biomass and shoot biomass than both controls. Like perennial wheatgrass, perennial wheat lines showed larger root mass than the wheat cultivar, especially at depth. At maturity, part of their leaves and stems were still green. These differences appear to be associated with maturity time and the strength of perenniality.

The regrowth ability of our breeding lines varies widely in different years or environments, which was also observed in investigations by other researchers (Tsitsin, 1978; Murphy *et al.* 2009; Hayes *et al.* 2011; Jaikumar *et al.* 2012). Extremely hot, cold, and dry weather conditions all can suppress the regrowth or cause the death of new tillers. We noticed that irrigation or rain after anthesis could promote regrowth. We have kept a number of lines in the greenhouse for continuous observation. The plants were watered every day, so that they were not stressed by moisture. However, the plants died with no more than three grain harvests. The number of tillers declined over life cycles. Unlike wheatgrass plants, the position in the crown where new tillers originated moved upward over time, which could make buds and new tillers vulnerable to environmental stresses. Furthermore, the new tillers entered the reproductive stage quickly without the need of vernalization. We have not found a line among our materials showing a typical winter-type regrowth profile in which the new tillers stay dormant like wheatgrass. Tsitsin (1978) suggested that winter-type regrowth was desirable for stronger perenniality. Common to these tested lines is that they carried about one set of 14 wheatgrass chromosomes.

An exception is MT-2, which was selected for forage production by Montana State University. In the Kansas environment, this hybrid line among all those we have obtained from other institutions is the most perennial. This line has lived in our field for two years up to present. It roughly contains two wheat genomes (mean = 26.2 wheat chromosomes) and two wheatgrass genomes (mean = 29.2 wheatgrass chromosomes) and is genetically unstable (Jones *et al*. 1999). The instability of this durum wheat (*T. turgidum* ssp. *durum*) hybrid and similar durum derivatives has led some to question the feasibility of durum as a parent of perennial wheat.

The problem of genetic instability may possibly be overcome by taking advantage of genetic variability within tetraploid species. Among the progenies derived from a cross of durum wheat with *Th. junceiforme*, we have found lines likely to be stable, predicted on the basis of seed fertility. Tetraploid wheat species may also possess genes that could promote genetic stability. It seems unnecessary to backcross with common wheat as advised by Ellneskog-Staam and Merker (2002).

By means of backcrossing to wheatgrass or intermating different perennial hybrid progenies at their early generations, we obtained 843 truly perennial plants that survived the hot Kansas summer in 2010 for the first time. These plants varied greatly in perenniality, seed fertility, vigour, regrowth habit, and other traits. Using the genomic *in situ* hybridization (GISH) technique, we examined the genomic constitutions of 11 plants. They possessed chromosome numbers ranging from 53 to 70, with some wheat chromosomes lost. In order to identify which wheat chromosomes might be associated with the elevated perenniality, we screened 154 wheat chromosome-specific markers (http://probes.pw.usda.gov:8080/snpworld/Search) on a panel of wheat and wheatgrass parents. We chose these markers because they had been assigned to 21 wheat chromosomes individually and had the capacity to distinguish homologous chromosomes. The presence of a marker clearly indicated the presence of a wheat chromosome. Fifty-six markers that amplified only in wheat were selected eventually to genotype a subset of 94 perennial plants (at F_2 to F_5 generations). The results showed that the patterns of wheat chromosome elimination differed by plants. All 94 plants had lost more than one wheat chromosome. With more wheat chromosomes eliminated, a plant tended to be more perennial. However, we did not see a specific wheat chromosome that was missing consistently across all plants, which would have indicated that a single wheat chromosome had a deciding impact on perenniality. It might be concluded that decreased abundance of wheat chromosomes is necessary for perennial wheat to live longer in the Kansas environment. Alternatively, the ratio of annual to perennial chromosomes may be critical to perenniality.

Cox *et al*. (2006) proposed that more than one wheatgrass genome would be required for perennial wheat to live in the field for many years. With the increased abundance of wheatgrass chromosomes and decrease in wheat chromosomes, some new issues need to be addressed. For instance, at present we do not know what percentage of wheatgrass chromosomes is appropriate for stronger perenniality that will ensure survival for multiple years. We also do not know if the decrease in wheat chromosomes will lead to more severe genetic instability. When polyploid wheatgrass species are used in crosses, wheatgrass chromosomes tend to be eliminated gradually in subsequent generations until a stable genomic constitution is reached (Tsitsin, 1978). It is impossible to assess the effects of complete genomes adequately using advanced generation materials due to chromosome elimination. To investigate these issues, we are establishing a panel of full amphiploids with different genomic constitutions that are composed of wheat: wheatgrass chromosome ratios of 3:3, 3:2, 2:3, 2:2, 1:3, and 1:2, respectively. Diploid, tetraploid, and hexaploid wheat species have been crossed with tetraploid and hexaploid wheatgrass species successfully. A fraction of such full amphiploids have been chromosome doubled, including the F_1 hybrid (2n = 84) between common wheat and *Th. intermedium* wheatgrass. Comparisons among them will help us understand how many wheat and wheatgrass genomes will enable perenniality across diverse environments.

The role of cytoplasm in the crosses of wheat with wheatgrass species may be worth exploring. Wide hybridizations in wheat and oilseeds have indicated that sequence deletion was different in reciprocal crosses, reflecting the interaction between nucleus and cytoplasm (Gill, 1991; Song *et al*. 1995; Ma and Gustafson, 2008; Ozkan and Feldman, 2009). To the best of our knowledge, all the existing perennial wheat lines bear wheat cytoplasm. We speculate that due to incompatibility with wheat cytoplasm, some wheatgrass chromosomes or genes important to perenniality are preferentially eliminated or silenced. This may be why all existing lines are not as persistent as the F_1 hybrids or perennial parents. Viable embryos are very difficult to obtain when wheatgrass is used as the female parent. Out of about 120 heads pollinated, we obtained a single plant with cytoplasm from *Th. intermedium*. We are investigating whether wheatgrass cytoplasm will aid the retention of wheatgrass chromosomes important to perenniality.

The easy crossability of wheat with *Thinopyrum* species provides opportunities for the exchange of genetic information between annual and perennial grass species. Both genera have their own pros and cons. We regard perennial wheat a hybrid species that bridges two genera. Putting more wheatgrass chromosomes/genes into wheat or vice versa appears to be the approach to 'truly' perennial wheat. As indicated by MT-2 and our recent investigations, the cross of durum wheat and *Th. intermedium* might be the best approach to explore in the coming years.

Perennial wheat breeding has been conducted for over 80 years. Hundreds of perennial wheat lines have been developed by different institutions. Certainly survival is dependent upon environmental variables, and agronomic practices developed specifically for perennial wheat may be critical to the persistence of perennial wheat. But declining stands in even the most moderate environments leads us to conclude that genetic improvement will be essential to persistence. The lack of sufficient persistence remains the biggest problem in the development of perennial wheat. The existing lines (with one set of wheatgrass chromosomes) have partly inherited the traits (e.g. large roots, tolerance to abiotic and biotic stresses) that make perennial wheatgrass persistent across varied environments. Before we have 'truly' perennial wheat, the use of these traits could yield some agronomic and ecological benefits for the time being. For instance, recent study of hybrid lines revealed novel disease resistance (Turner *et al*. 2013). Although current lines lack perenniality, their grain traits are similar to wheat. Therefore, these types could be economically viable in the near term while reliably perennial wheat varieties are developed.

Intermediate Wheatgrass Breeding in Kansas

Selection at the Land Institute has been based on individual plants spaced 0.91 metres apart. The first selection cycle comprises 1 000 clones replicated three times. The second selection cycle used about 4 000 individual plants. After two cycles of selection primarily for seed yield per head and seed mass, the resulting populations were planted in a replicated solid-seeded trial at two locations. For controls, the base population (consisting of seed obtained from the Rodale Institute) and several forage varieties were used. For simplicity we are presenting data from the irrigated location in the second year of production.

Two cycles of selection resulted in a 77 percent increase in seed yield (Table 1). If progress were to continue in a linear manner, an additional 12 cycles of selection would result in yields of approximately 2 500 kg ha^{-1}, similar to annual wheat in Kansas. Since this evaluation was initiated, an additional two cycles of selection have been performed, requiring two years per cycle. Therefore, we expect that with sustained efforts yields in Kansas may be similar to wheat within 20 years.

Seed size has responded weakly to index selection in space plants, when evaluated in a solid stand (Table 1). If trends from the first two cycles continue, about 110 years of sustained effort would be necessary to achieve a seed size of 30 mg $seed^{-1}$, similar to annual wheat. Therefore, large seed size may only be attained by using other approaches. We are currently beginning efforts to introgress genes controlling seed size from wheat. Also, genomic approaches may be useful in screening diverse collections for alleles conferring increased seed size.

TABLE 1. PERFORMANCE OF INTERMEDIATE WHEATGRASS POPULATIONS BEFORE (CYCLE 0) AND FOLLOWING ONE (CYCLE 1) AND TWO (CYCLE 2) CYCLES OF SELECTION

Three intermediate wheatgrass cultivars (Luna, Manska, and Rush) and one tall wheatgrass cultivar (Jose) are included as controls.

	SEED YIELD	BIOMASS YIELD	SEED MASS	HARVEST INDEX	CP[a]	NDF[a]	ADF[a]
	g m^{-2}		mg $seed^{-1}$	%	g Kg^{-1}		
Cycle 0	39.2	1 770	3.92	2.21	37.4	687	465.3
Cycle 1	56.5	1 920	4.48	2.94	30.6	680	461.1
Cycle 2	69.3	1 740	4.84	3.93	33.5	662	444.5
Luna	17.8	1 480	4.10	1.22	45.0	630	447.5
Manska	21.6	1 610	3.41	1.35	37.0	653	435.7
Rush	26.3	1 730	3.61	1.55	34.5	655	440.6
Jose	6.6	1 500	4.06	0.46	44.7	683	478.7
SEM[b]	7.4	120	0.15	0.39	3.0	11	8.2

a Crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined for the non-seed biomass collected at seed maturity.

b Standard error of the mean.

Biomass production has remained mostly unchanged during selection for seed traits (Table 1). Therefore, rising grain yields are apparently occurring due to increased harvest index. In fact, across the populations evaluated the correlation between grain yield and harvest index was 0.996.

Forage quality indicators of the biomass residue were measured to determine whether selection for seed production was reducing forage quality (Table 1). So far the effects of selection have been minor relative to forage varieties, at least when measured at the late stage when seed is ripe. However, we expect that increased allocation of nitrogen to seed will ultimately reduce protein content of the residue. For systems involving both grain and forage production, selection for forage quality at seed maturity may be necessary.

During the third cycle of selection in space-planted nurseries, several individuals with outstanding yield per head were identified. A second set of plants with large seed size were also selected. These two groups of plants were cloned by dividing their crowns and used to establish bordered plots 0.91 metres square at two locations: Kansas and Minnesota. The Kansas location has higher summer temperatures, but was irrigated. The Minnesota location was not irrigated. Although the plants were selected in the Kansas environment, difference in seed yield between selected and unselected types was larger at the more moderate Minnesota location (Table 2). Total aboveground biomass was similar among genotypes, and higher than potential perennial biofuel crops for this region (Parrish and Fike, 2005). In Minnesota, the largest-seed genotypes had seed yield more than 200 g/m^2 (Table 2). These yields were obtained from the best genotypes in small hand-harvested plots, but they indicate that there is potential to obtain substantial seed yields from this perennial grass.

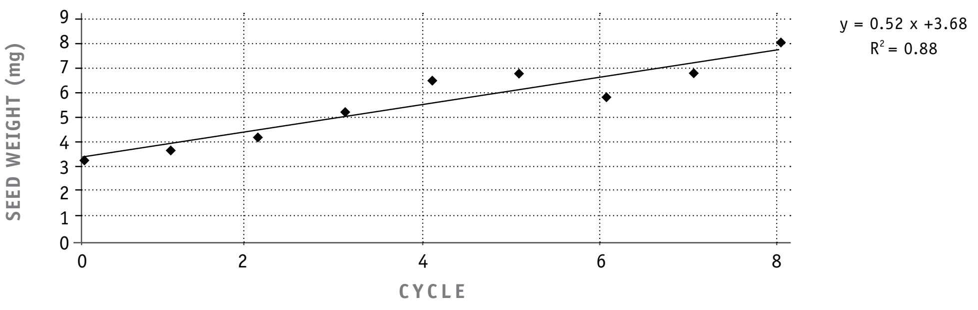
TABLE 2. PERFORMANCE OF INTERMEDIATE WHEATGRASS AT TWO LOCATIONS IN THE SECOND YEAR

Sets of clones (seed weight and seed yield) were compared with the starting population (Cycle 0) and a forage cultivar (Clarke). Predicted means from a mixed model, ± standard errors, are presented.

POPULATION	BIOMASS				SEED YIELD				SEED WEIGHT			
	MINNESOTA		KANSAS		MINNESOTA		KANSAS		MINNESOTA		KANSAS	
	g m^{-2}								mg $seed^{-1}$			
Cycle 0	1 690	±160	1 650	±160	84	±14	68	±14	5.73	±0.39	4.10	±0.39
Clarke	2 000	±160	2 170	±160	117	±14	49	±14	5.10	±0.39	3.73	±0.39
Seed Weight	2 380	±220	1 660	±110	212	±19	88	±10	9.26	±0.87	7.57	±0.66
Seed Yield	1 830	±140	1 810	±100	192	±12	100	±9	7.60	±0.68	6.07	±0.62

Since 2003 we have been performing mass selection for seed size. Initially, we began by harvesting a set of plants and obtaining the naked seed by floating off seed remaining in hulls. The naked seed was then passed through a set of sieves to obtain the largest seeds. These were planted, and then the process was repeated in the following year. After three cycles of selection, we began using a machine to automatically weigh each naked seed and sort out the largest seeds. We became concerned that this selection method might cause increased see sterility, since seed number and size are often inversely correlated (Sadras, 2007). So beginning with the fifth selection cycle we threshed each plant separately to obtain a seed yield per plant. Seed from the lowest-yield plants was discarded prior to sorting out the largest naked seed for selection. The selection intensity has varied from year to year, depending on seed yield. But in the later selection cycles about 120 plants have been established each cycle, selected out of about 40 000 naked seeds harvested. Seed size has fluctuated over the years depending on growing conditions and selection protocol. But the trend of increasing seed size has been strong (Figure 1). Eight cycles of selection have more than doubled seed weight when grown in a spaced plant selection nursery.

FIGURE 1. RESPONSE OF INTERMEDIATE WHEATGRASS TO MASS SELECTION BASED ON INDIVIDUAL SEED WEIGHT



Intermediate wheatgrass breeding in Manitoba

In the spring of 2011, a 4 500 plant nursery was established at the Carman, Manitoba research farm of the Department of Plant Science at the University of Manitoba. Accessions from the Germplasm Resources Information Network (GRIN) system of the United States Department of Agriculture (USDA) plus advanced generation materials from the Land Institute were planted.

The winter of 2011 to 2012 was relatively short with snowfall arriving in early December 2011 and snowmelt completed by 13 March 2012 on our Carman, Manitoba plots. For six days beginning on 19 March 2012, the daytime high was at least 19.9°C on four days with low temperatures above 0°C for the six days. This was followed by a four day period beginning 9 April 2012 where low temperatures were -5.5°C or lower. Approximately 50 percent of the 4 500 plants in the field sustained severe die-back. An additional 15 percent lodged prior to flowering, mostly due to reduced crown density caused by low temperature damage. The winter of 2012 to2013 was by comparison long and cold. Overnight lows below 0°C began on 31 October 2012 and lasted until 25 April 2013. Snow cover remained until 6 May 2013. An additional 15 percent of the remaining plants were lost due to these winter conditions. We have now experienced two sets of vastly divergent winter conditions and we now feel that we have excellent materials from which to base our selections. Yield stability will be evaluated utilizing the yields from the first two harvests (2012 and 2013). In 2013, some plants were removed from the nursery due to visually high levels of ergot.

Selection has taken place on the materials received from The Land Institute. A subsample of 100 plants was chosen at random to encompass the range of plant morphology within the nursery to investigate yield and its components. A comparison of the two sources indicates that the increased grain production has resulted in a higher harvest index (Figure 2). This has in part been achieved by a reduction for reduced spread (Figure 3). A major result of selection appears to be the increase in the seed yield per unit area of the plant (Figure 4). Initial selection will be based upon consistency of yield between years with an emphasis towards high yield per unit area. This would appear to lead towards a reduced plant size (at least plant area) and potentially higher yields in solid-seeded plantings.

Plant establishment greatly impacts competitiveness. The ability to rapidly emerge from the soil and compete with other species will impact productivity. Six half-sibling families were selected to study the effect of seed size on germination and emergence.

FIGURE 2. RELATIONSHIP BETWEEN HARVEST INDEX AND SEED YIELD PLANT^{-1} IN IMPROVED AND NON-IMPROVED INTERMEDIATE WHEATGRASS

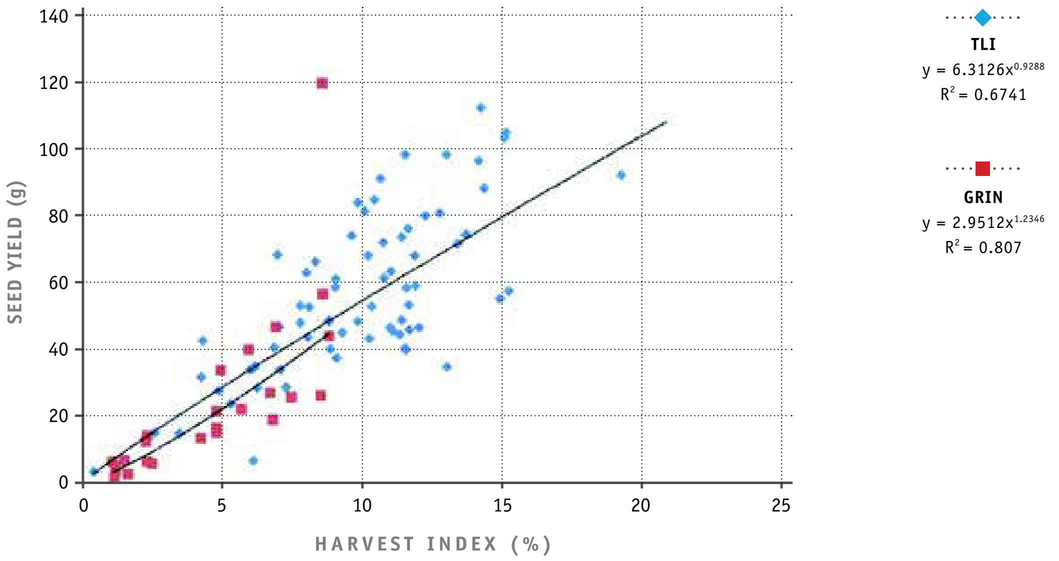


FIGURE 3. RELATIONSHIP BETWEEN PLANT AREA AND SEED YIELD PLANT^{-1} IN IMPROVED AND NON-IMPROVED INTERMEDIATE WHEATGRASS

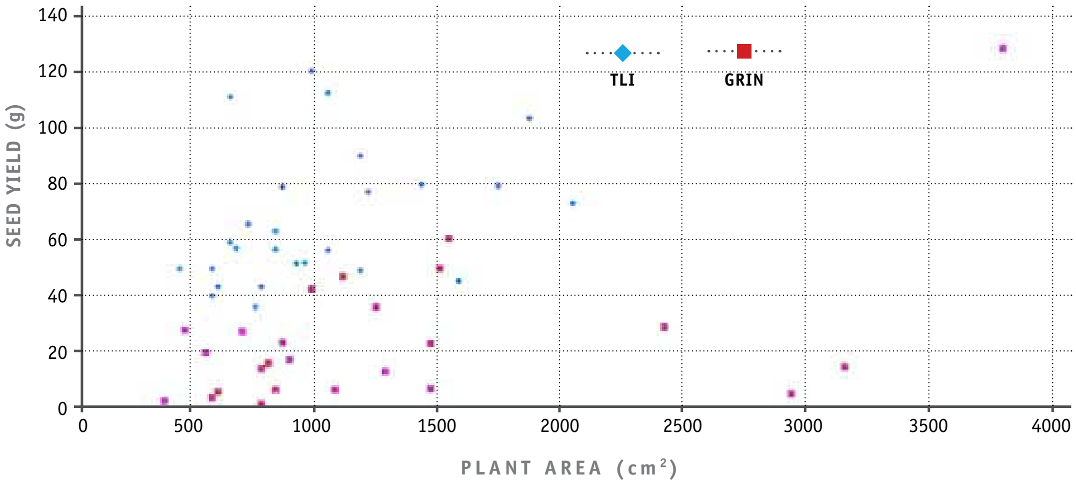


FIGURE 4. RELATIONSHIP BETWEEN PLANT AREA AND SEED YIELD CM^{-2} OF PLANTS IN IMPROVED AND NON-IMPROVED INTERMEDIATE WHEATGRASS

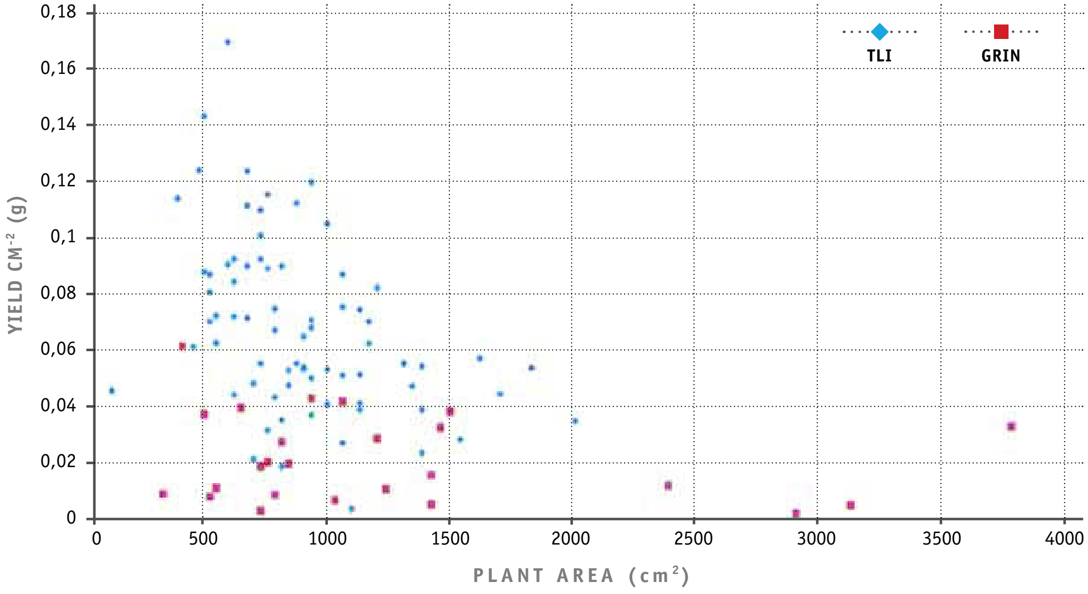
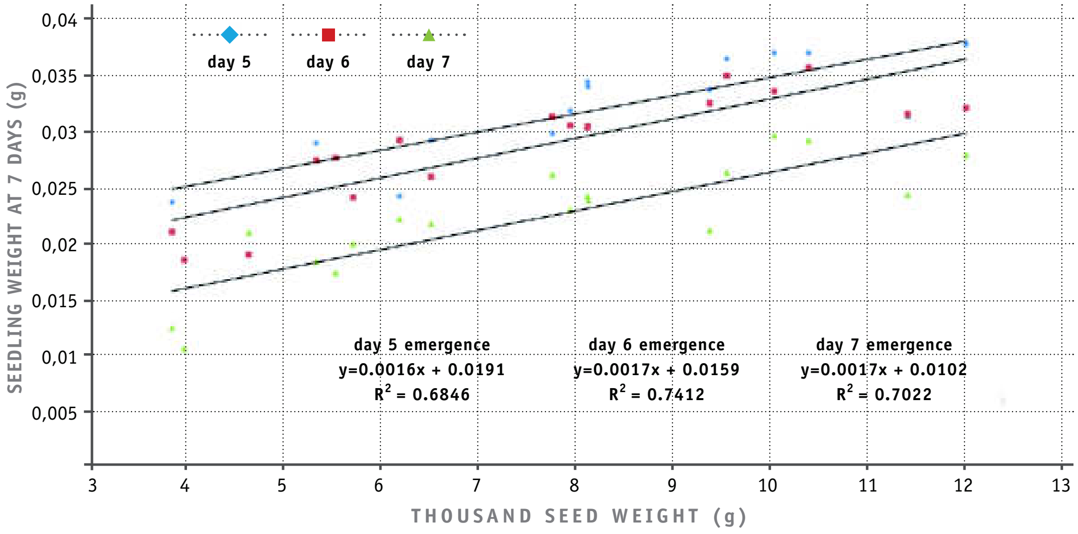


FIGURE 5. RELATIONSHIP BETWEEN THOUSAND SEED WEIGHT AND INITIAL BIOMASS ACCUMULATION IN INTERMEDIATE WHEATGRASS IN THE SEVEN DAYS AFTER EMERGENCE



Germination tests were conducted to ascertain seed viability, and emergence tests from a 2 cm depth (both field and greenhouse) were used to study relative ability to establish. Correlation between the germination tests and greenhouse emergence was high ($r = 0.901$), while emergence in the greenhouse and field was $r = 0.781$. Field emergence and germination tests showed the lowest correlation ($r = 0.623$). Emergence was not necessarily related to seed weight and the ability of plants to amass dry weight was relatively uniform for the first three days of emergence (Figure 5). The earlier the emergence, the greater the seedling weight after seven days of growth (Figure 5). This may be attributed to less energy being expended during emergence, thus resulting in a higher intercept value. Alternatively, early-emerging seedlings may possess genes contributing to rapid growth both before and after emergence.

Improving intermediate wheatgrass quality

Intermediate wheatgrass grain is currently inferior to wheat for most potential uses. Many of the limitations are due to a small grain size and the resulting high fibre and protein of whole grain flour. Small seed produces a low milling yield of white flour. Small grain size is being addressed through breeding, but an additional limitation is the low gluten quality that limits utility in raised breads. We have found that the low gluten quality of intermediate wheatgrass may be due to a very low quantity of HMW-GS.

In fact, HMW-GS genes have been transferred from intermediate wheatgrass to wheat to improve quality (Li *et al.* 2013; Niu *et al.* 2011). Therefore, we investigated the diversity and potential function of HMW-GS alleles in intermediate wheatgrass. Using gene cloning, protein separation and identification, and sequence alignments, we were able to identify five HMW-GS genes and their allelic variants in intermediate wheatgrass plants. Because the species is diverse and heterozygous, one or two allelic variants of each gene were detected in each plant, and an average of five HMW-GS proteins were found in individual plants.

Protein chemistry has demonstrated that all the identified HMW-GS were involved in protein polymers. Sequence alignments showed that two genes share high identities (>96 percent) with the HMW-GS genes from the D-genome of common wheat. We predict that with selection for high expression of superior allelic variants, HMW-GS might contribute to the bread-making quality of intermediate wheatgrass.

Intermediate wheatgrass molecular work

EST markers were developed for allohexaploid intermediate wheatgrass (*Thinopyrum intermedium*) and three closely related diploid species *Pseudoroegneria spicata, Thinopyrum bessarabicum*, and *Thinopyrum elongatum* (Table 3).

TABLE 3. DESCRIPTION OF ESTS AND EST MARKER RESOURCES FOR INTERMEDIATE WHEATGRASS

	P. spicata	*Th. intermedium*	*Th. elongatum*	*Th. bessarabicum*
Platform	Sanger	Roche 454	Roche 454	Roche 454
Reads (avg. bp)	16 128	566 681 (432)	447 936 (421)	364 193 (426)
Contigs (avg. bp)	6 450 (1 017)	29 851 (637)	30 858 (650)	15 700 (701)
Singletons (avg. bp)	2 330 (670)	45 760 (374)	38 216 (365)	22 709 (348)
EST isotigs (avg. bp)	8 780 (924)	75 611 (478)	69 074 (492)	38 409 (492)
Total bp	8 120 750	36 153 671	34 024 034	18 928 290
EST-SSR markers	1 375	672	596	384
Hv1[a]	152	98	70	68
Hv2	185	109	87	45
Hv3	172	106	90	54
Hv4	176	100	87	55
Hv5	204	98	95	77
Hv6	158	70	76	41
Hv7	187	91	71	44

[a] Correspondence of EST-SSR markers to the seven barley chromosomes (Hv1 to Hv7).

It is thought that the three subgenomes of allohexaploid intermediate wheatgrass may include one *Pseudoroegneria* (**St**) genome and two *Thinopyrum* (**E**) genomes similar to *Th. elongatum* ($\mathbf{E}^{e}$) and/or *Th. bessarabicium* ($\mathbf{E}^{b}$) genomes (Liu and Wang, 1993; Zhang *et al.* 1996; Zhang *et al.* 1997). A total of 1 375 Pspi EST-SSR primer pairs were previously developed from *P. spicata* (Table 3) (Bushman *et al.* 2008). Another 1 652 EST-SSR markers were recently designed from Roche 454 ESTs from intermediate wheatgrass, *Th. elongatum*, and *Th. bessarabicium* (Table 3). All of the *Thinopyrum* EST-SSR markers were designed from ESTs corresponding to one of the seven barley (Hv) chromosomes (Table 3). Likewise, most of the previously designed *P. spicata* EST-SSR markers also correspond to one of the seven barley (Hv) chromosomes (Table 3) based on alignments to the barley genome sequence (The International Barley Genome Sequencing Consortium, 2012).

The *P. spicata* and intermediate wheatgrass EST-SSR primers have been tested for amplification and/or polymorphism in intermediate wheatgrass and related species. Approximately 79 percent (1 083) of the *P. spicata* EST-SSR primers amplified products from *P. spicata* (Bushman *et al.* 2008). More recent experiments also showed that about 80 percent (1086) of the *P. spicata* EST-SSR primers amplified products from both *P. spicata* and intermediate wheatgrass. Similarly, about 84 percent (564) of the intermediate wheatgrass EST-SSR primers amplified products from intermediate wheatgrass. Moreover, about 68 percent of the intermediate wheatgrass EST-SSR primers amplified products from Chinese Spring wheat.

A combination of EST-SSR and AFLP markers will be used to genotype an experimental mapping population comprises 268 full-sib progeny derived from reciprocal crosses of two experimental genotypes, M35 × M26 (137) or M26 × M35 (131). The M26 genotype derives from a cross of C3-3471 × C3-3941. The C3-3941 genotype was also the female parent of M35. So far, six AFLP primers have also been genotyped on M26, M35, C3-3471, C3-3941 and 268 full-sib mapping progeny. These six AFLP primers detected 133 AFLP markers that were polymorphic between M35 and M26 with an average marker frequency of 0.52 among the 268 progeny. These six AFLP primers also detected 75 AFLP markers that were present in both M35 and M26 with an average marker frequency of 0.76 among the 268 progeny. Thus, a total of 208 polymorphic markers showing expected 1:1 or 3:1 segregation ratios were detected using the first six AFLP primer pairs. Another 13 AFLP markers that were present in both M35 and M26 showed marker frequencies greater than 0.9, which could be the result of segregation distortion or non-disomic inheritance. However, initial results were largely consistent with disomic inheritance. Although the mapping population has not yet been genotyped with EST-SSR markers, the M26, M35, C3-3471, and C3-3941 parents and grandparents have been screened using the 672 intermediate wheatgrass EST-SSR markers. Nearly 17 percent of the 564 amplified markers showed clear polymorphism between the M26 and M35 parents of this intermediate wheatgrass mapping population. Thus, we estimate that approximately 440 EST-SSR markers will be informative if this rate of polymorphism applies to *P. spicata*, *Th. elongatum*, and *Th. elongatum* EST-SSR markers.

Genotyping-by-sequencing (GBS) combines molecular marker discovery and genotyping and has been successfully used in species with large, complex genomes, such as barley and wheat. Intermediate wheatgrass is an allohexaploid with an estimated 14 GB genome and no reference genome. The combination of a large and understudied genome makes genetic studies and genomic assisted breeding for intermediate wheatgrass challenging. To address these constraints, we are utilizing the GBS approach to discover SNPs, and then using them to construct a genetic map and map important agronomic traits.

GBS involves simultaneously sequencing DNA from many individuals, but only specific sites in the organism's genome. The GBS approach increases the sequence coverage per site and dramatically reduces the sequencing cost per individual. These specific sites are targeted by restriction enzymes; for intermediate wheatgrass, we are using the restriction enzyme *PstI*, which identifies all sites in the genome with the DNA sequence 'CTGCAG' and cuts the DNA between the A and G nucleotides. A DNA barcode (unique 5-10 bp sequence) and barcode adapter are attached to the DNA fragments from each individual. DNA from many individuals is then pooled and sequenced as if it were from one individual (we sequence 96 individuals at a time). DNA is currently sequenced using Illumina's HiSeq platform and SNPs are called using the Universal Network Enabled Analysis Kit (UNEAK) bioinformatics pipeline.

The genetic map analysis began with ~3000 high quality SNPs from 285 selfs, part of a larger population of ~750 selfs from one individual. Intermediate wheatgrass is primarily outcrossing, but a large effort was concentrated on selfing an individual with an important set of agronomic traits: free-threshing, large seed and non-shattering. Preliminary phenotypic analyses suggest that these traits are correlated, dominant, and segregating in the selfed population. The framework map has been constructed with MapMaker and includes 859 SNPs from 271 individuals, divided into 22 linkage groups (there are 21 chromosomes). One small linkage group shows linkage with two larger linkage groups, but cannot be precisely placed. Preliminary analyses suggest that GBS can be utilized in intermediate wheatgrass for *de novo* genotyping to develop high-density genetic maps and genomic selection models for crop improvement.

Genetic studies of intermediate wheatgrass are complicated by the plant's high degree of heterozygosity, hexaploid nature, and self-incompatibility. Future work would be simplified by access to completely inbred individuals. We have been attempting to obtain fully homozygous individuals by producing doubled haploids through another culture. So far approximately 20 000 anthers have been cultured. Hundreds of calluses have formed. Dozens of calluses have initiated shoots, but most have been albino. We have obtained 5 plantlets that are green. The haploid nature of three young plants has been confirmed, and we are attempting to double their chromosome numbers with colchicine.

CONCLUSIONS

"Perennial wheat" hybrids and intermediate wheatgrass are currently at far ends of a spectrum. While the hybrid types have good grain yield, large seed, and other favourable traits, they remain functionally annual in most environments. On the other hand, intermediate wheatgrass is a vigorous perennial that is easily grown in diverse environments. But it lacks adequate seed yield, seed size, and grain quality to be commercially successful. Working from both directions, we expect that these programs will meet somewhere in the middle. Along the way, much of what is learned and developed in one approach will be useful to the other. For instance, genetic maps developed for intermediate wheatgrass will be critical for perennial wheat development. Understanding the physiology, morphology, phenology, and genomic functioning of perennial wheatgrass will be useful to those developing perennial wheat. Similarly, much that is known about the quality, pathology, yield, and genomics of wheat is being readily applied to intermediate wheatgrass.

We should not choose between the strategies of wide hybridization or domestication in the development of perennial small grains. The most rapid progress will be made by simultaneously working from both ends of the spectrum in order to produce perennial grain crops. Whether the perennial grain of the future will be wheat with grass-like traits added or a perennial grass with the addition of wheat-like traits is a question that we need not dwell on. What is clear is that progress is being made at an accelerating pace toward the day when useful, high-yield, long-lived small grains are a reality.

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