Trends in Plant Science

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REVIEWS

Opinion

Roadmap for Accelerated Domestication of an Emerging Perennial Grain Crop

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Shifting the life cycle of grain crops from annual to perennial would usher in a new era of agriculture that is more environmentally friendly, resilient to climate change, and capable of soil carbon sequestration. Despite decades of work, transforming the annual grain crop wheat (*Triticum aestivum*) into a perennial has yet to be realized. Direct domestication of wild perennial grass relatives of wheat, such as *Thinopyrum intermedium*, is an alternative approach. Here we highlight protein coding sequences in the recently released *T. intermedium* genome sequence that may be orthologous to domestication genes identified in annual grain crops. Their presence suggests a roadmap for the accelerated domestication of this plant using new breeding technologies.

Highlights

Current grain crops are annuals that must be sown every year, giving their root systems little time to develop during the growing season.

A perennial grain crop with a long-lived extensive root system would improve soil quality, store carbon belowground, and utilize water and minerals more efficiently.

Domestication genes of the annual grass wheat are highly conserved in the perennial intermediate wheatgrass (*Thinopyrum intermedium*), providing an opportunity for accelerated domestication of a perennial grain using a mutagenesis approach.

A Candidate Perennial Grain Crop

Crop production is facing unprecedented challenges. In 2050, the human population will likely exceed nine billion [1], increasing the demand for staple crops and livestock by 60% [2]. Climate change is expected to drastically constrain plant productivity, necessitating the development of cultivars with increased tolerance to abiotic stresses such as heat, drought, soil salinization, and flooding. Furthermore, yields are beginning to stagnate in important crop production regions [3]. Therefore, a movement is building to intensify crop production sustainably through the development of new crop species [4].

Jackson [5] proposed that maximum sustained crop yields could be achieved through developing perennial grain crops. Worldwide, annual grain crops provide about 70% of human caloric needs and occupy about 70% of crop lands [6]. Annual crops must be sown every year, which disturbs the soil and exposes it to erosion through tillage or clearing of vegetation with herbicides. Furthermore, in the beginning of the growth season, the shallow root systems are inefficient at taking up water and nutrients, which is a major cause of ground and surface water pollution by nitrate leaching. A perennial grain crop that does not need to be sown every year would develop a long-lived deep root system (Figure 1A) that sequesters carbon and takes up nutrients and water efficiently [7,8]. This crop could be intercropped with perennial legumes to provide additional ecosystem services such as nitrogen fixation [9]. Furthermore, the large root systems, storage reserves, and stress tolerance of perennial ancestors in a perennial grain **domestication** (see Glossary) program would provide abundant mechanisms for developing new crops tolerant to a wide array of stresses [10].

Attempts to develop perennial wheat began in the 1930s, but have so far not produced a widely grown cultivar [11]. In 1988, a Eurasian forage grass called intermediate wheatgrass (*Thinopyrum intermedium*), which is a close perennial relative of wheat, was selected as a promising perennial grain candidate at the Rodale Institute [12]. The intention was to create a productive grain crop through phenotypic selection within the species. Two cycles of breeding for improved grain

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Figure 1. Comparison of Intermediate Wheatgrass (IWG; *Thinopyrum intermedium*), a Perennial Grass Species, with Wheat, an Annual Grass. (A) The extensive perennial root system of intermediate wheatgrass grows to more than twice the depth of annual wheat in a long-column pot with artificial medium. (B,C) IWG is crosspollinated, producing large protruding stamens (B) that shed abundant wind-borne pollen (C). (D) Comparison of spike morphology of hard red winter wheat (right) and IWG (left). A shattered head with seed that was shed by gentle brushing is shown at the far left. (E) IWG seed in-hull (top), dehulled IWG seed (middle), and hard red winter wheat (bottom). The average mass of dehulled IWG seed shown was 10 mg seed^{-1} and the mass of wheat seed was 36 mg seed^{-1}. (F) Cut wheat seeds (left) and IWG seeds (right) showing differences in seed plumpness.

Glossary

Accelerated domestication: the process of mutating genes in a wild plant that resemble domestication genes in a known crop with the purpose of domesticating the wild plant.
Base editing: methods that change a specific DNA nucleotide into another.
Base editor: a molecule used for base editing. Typically, a modified Cas9 variant nuclease is used that is fused to a deaminase that converts one base to another along with single guide RNA (sgRNA) that directs the fusion protein to the target sequence.
CRISPR-Cas9: a method to introduce targeted double-strand breaks in DNA. In most cases, the plant cells will be able to repair the break, but sometimes mistakes occur and a mutation results. Mutations obtained using this method are often small insertions or deletions (indels).
***De novo* domestication:** the process of mutating domestication genes in a wild ancestor of a crop with the purpose of domesticating the crop again from scratch.
Domestication: the process of selecting for variants in plant populations that serve human needs (e.g., have increased yield, better taste, or reduced toxicity). The variants are typically less fit in nature than their ancestors and depend on human care to survive.
Domestication gene: a gene that when mutated in a wild plant contributes to a domestication phenotype (e.g., nonshattering).
Genome editing: methods that introduce targeted mutations in a plant genome that cannot be distinguished from spontaneous mutations appearing in nature.
Homoeologous chromosomes: in allopolyploid plants, homoeologous and homologous chromosomes are different in that homologous chromosomes normally pair and recombine during meiosis, whereas homoeologous chromosome do not normally pair and recombine during meiosis. Allohexaploid plants such as wheat and *T. intermedium* evolved by hybridization of three distinct species and the fusion of three related genomes each having seven homologous chromosome pairs. Accordingly, the 21 chromosome pairs of wheat or *T. intermedium* can be divided into seven homologous pairs for each of three homoeologous chromosomes or three homoeologous

production were completed in New York, USA, between 1990 and 2000 [13]. The Land Institute's domestication program for intermediate wheatgrass began in 2003 and continues to the present time [13]. Eight generations of selecting and intermating the best plants based on their yield, seed size, shatter resistance, and other traits have been performed, resulting in improved populations of *T. intermedium* that are currently being evaluated and further selected at The Land Institute and by collaborators in diverse environments. However, these breeding approaches have not yielded *T. intermedium* varieties that would be profitable for farmers to produce at large scale. On-farm yields of *T. intermedium* varieties are currently less than 20% of that of wheat and seed mass is about 25% that of wheat seeds. The breeding program is currently focused on selecting for several traits, including yield, shatter resistance, free threshing ability, seed size, and grain quality. Although progress is steady, the urgent need to sustainably boost food production necessitates the development of methods to dramatically accelerate the pace of domestication.

groups of seven homologous chromosome pairs.
Homoeologous groups: groups of **homoeologous chromosomes** that diverged through speciation in evolutionary history but have now been brought back within the same genome through allopolyploidization.
Ortholog: orthologous genes are genes from different species or genomes that evolved from the same gene and have the same function. Allopolyploid plants may have two or more copies of the same orthologous gene, known as homeologs, originating from hybridization of two or more ancestral species.

Challenges for Future Breeding Approaches

A high-quality genome sequence of *T. intermedium* (available at https://phytozome-next.jgi.doe.gov/info/Tintermedium_v2_1), which promises to facilitate future breeding approaches, was released in early 2019 by the US Department of Energy Joint Genome Institute. Like wheat, it has a large and complex allohexaploid genome ($2n = 6x = 42$) with three genomes in one and containing approximately 11 912 million nucleotide base pairs. Given the near-obligate outcrossing nature of *T. intermedium*, allelic diversity is very high in every population studied so far. Therefore, no population will be genetically identical to the reference genome. Because the genome is hexaploid, six alleles of a given gene may be present in a single individual. In a larger population, many allelic variants are expected at every locus. This inherently large diversity makes it extremely challenging to identify mutants by phenotypic evaluation. Novel variation induced by mutagenesis and already present genetic variation can be identified by target induced local lesions in genomes (TILLING) and ecotype TILLING (EcoTILLING) approaches, respectively [14]. If a recessive mutation is required to produce a desired phenotype, each of the six alleles might need to be mutated. Therefore, searches for new promising recessive alleles may be futile if they are based on the phenotype of mutant plants. An alternative, and perhaps the only feasible way to search for useful variation, is to locate variation directly at the DNA level in the genome. In the case of recessive mutations, one would need to identify mutations in all three genomes and then breed to obtain homozygosity at all three loci. This would have to be accomplished through marker-assisted breeding and selection.

There is substantial divergence between the three genomes of *T. intermedium* and in many cases large deletions in gene **orthologs** suggest that many of the alleles are already nonfunctional. Therefore, it may be possible to identify instances where mutations are only needed in one of the three genomes in order to produce a desired phenotype. However, to design a working strategy, one would need to sequence every allelic form present in the individual to be mutated.

T. intermedium is primarily outcrossing (Figure 1B,C), which means that any seed pool will be heterozygous and heterogeneous. Distinguishing new mutations from existing mutations in such material is challenging. However, self-pollination for one or two generations is possible. Selfing could likely be used to obtain homozygosity at particular loci if these were tracked with markers, but it has not yet been possible to breed to full homozygosity. The sequenced individual used for the reference genome was a spontaneous and extremely rare haploid.

In our studies, *T. intermedium* has had a low tolerance to the mutagen ethyl methanesulfonate (EMS), with 100% mortality resulting from the typical dosage of 0.8% (v/v) used in wheat [15].

Furthermore, a transformation protocol for *T. intermedium* has not yet been developed, posing a major constraint to future **genome editing** approaches.

The Molecular Background for Domestication of Grain Crops

Domestication, the reiterative selection of plants with a desired trait, has resulted in genetic changes that distinguish domesticated taxa from their wild ancestors in traits such as shatter resistance, ease of threshing, and seed dormancy [16]. Molecular genetic studies have revealed several **domestication genes** in plants (mainly encoding transcription factors), loci underlying crop diversity (mainly encoding enzymes and structural proteins), and mutations that result in *cis*-regulatory changes [17–19]. These changes can affect the spatiotemporal expression patterns of genes or result in the complete or partial loss of function of domestication genes. For example, seed harvest has been facilitated by the neutralization of genes that promote seed shattering.

Two major hypotheses have been proposed to explain the molecular mechanisms of domestication: the genetic tinkering hypothesis (i.e., slow gain of new functions) posits that domestication proceeds via the modification of genes and physical features that are already present rather than the addition of fundamentally new characteristics or the loss of existing ones [20,21]. The 'tinkering' aspect of this process may resemble that of the evolutionary adaptation found in nature [20]. In support of this model, few genes that contributed to the domestication of diploid and ancient polyploid species are null alleles; gene mutations often cause altered protein function and/or gene expression rather than eliminate protein function. For example, ***de novo* domestication** of wild relatives of tomato (*Solanum lycopersicum*) was possible by altering protein expression through **CRISPR-Cas9**-assisted modification of *cis*-regulatory regions or upstream open reading frames [22].

The second hypothesis is the genetic disassembling hypothesis (i.e., rapid loss of function), which posits that a major portion of phenotypes associated with domestication, even those associated with gain of function and/or unaffected protein function, result from loss-of-function ('crippling') mutations (reviewed in [23]). Loss-of-function is not restricted to proteins but can also be loss or weakening of *cis*-regulatory mechanisms that help the plant survive in nature. Whereas these mutations are generally recessive, they can also be dominant (e.g., if they result from the loss of repressor sites in promoter regions). Furthermore, in polyploid species, null mutations of one homologous gene copy may have only subtle dosage effects and so appear as 'tinkering' mutations. Thus, domestication by 'crippling' resulted in the loss of many properties essential for plant survival in the wild and is consistent with the observation that domesticated plants are generally completely dependent on humans and can no longer compete in nature. Loss-of-function mutations that occur in coding regions often have profound pleiotropic effects, which weaken the plant overall. One solution that has been proposed for new domestications is to prioritize mutations in *cis*-regulatory mutations, which have more subtle effects and can produce the desired phenotype without weakening the organism in other ways [24].

It has been hypothesized that domestication is caused by changes in only a few domestication genes and that these events can be mimicked by mutagenesis of homologous genes in wild plants [23,25,26]. The phenotypic changes involved in domestication often have parallel genetic underpinnings, where mutations in homologous loci underlie the same phenotype across species [19]. Recent work has demonstrated *de novo* domestication of wild relatives of tomato (*S. lycopersicum*) by introducing mutations in the form of small insertions or deletions (indels) in as few as five genes, providing strong support for the genetic disassembling hypothesis [22,27]. The general applicability of these findings remains to be tested in a range of plant species. However, if this approach would allow for **accelerated domestication** of wild plants, it could have enormous potential for agriculture [28,29].

Recently, the orphan crop groundcherry (*Physalis pruinosa*) was partially domesticated by mutating orthologous domestication genes of tomato [30]. The genome of *T. intermedium* is closely related to that of wheat [31–33], which suggests that domestication genes in wheat are represented by orthologous genes in *T. intermedium*. Below, we will capitalize on this knowledge and expand on it to provide a roadmap for accelerated domestication of *T. intermedium* (Figure 2).

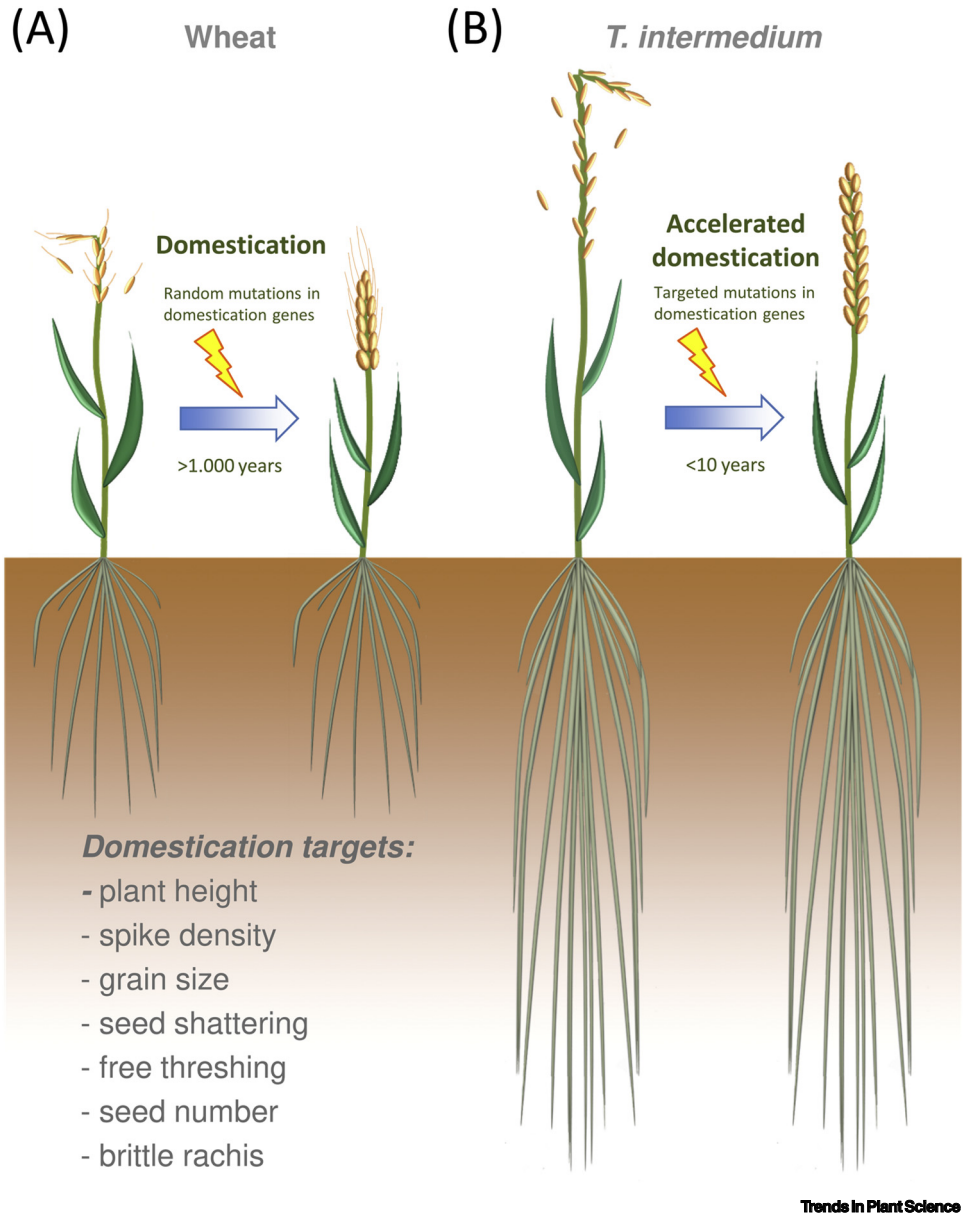

Figure 2. Domestication Targets in Wheat and *Thinopyrum intermedium*. (A) The wild ancestor of wheat (left) dispersed its thin grains readily and had low yields. Modern varieties of wheat (right) resulted from thousands of years of domestication. They have thick grains that stick to the spike but are threshable and the stem is shorter than that of its ancestors, so it tolerates the weight of the spike without lodging. (B) The perennial grass *T. intermedium* (left) lacks the same domestication traits absent in the wild ancestor to wheat. New breeding technologies may make it possible to accelerate the domestication of this cereal by directly targeting genes homologous to those that arose randomly and were fixed during the domestication of wheat.

A Possible Roadmap

Many domestication genes have been discovered in a variety of crop plants. Here we will focus on some that govern traits that were essential for the early domestication of grasses. During the process of crop domestication, human selection resulted in plants with larger seeds that were easier to harvest. Most wild grasses have abscission layers that cause the heads to break into smaller fragments that disperse their propagules. This seed shattering process is essential for survival of wild plant species, but would generate enormous losses in agriculture. Hence, one of the most important events in crop domestication has been the elimination of the natural mode of propagule dispersal. Two main natural modes of dispersal exist: (i) fruits separate (abscise) from the plant upon ripening, thus allowing seeds to come into contact with the soil; (ii) fruits remain attached to the plant, but open (dehisce) to release the seeds, which in turn abscise from the mother tissues. Abscission of fruits or seeds is dependent on the mechanical properties of the relevant tissues, and several domestication genes involved in seed shattering were shown to control lignification or the development of abscission layers [34].

Wild type *T. intermedium* plants exhibit seed shattering and most seeds remain attached to a hull (Figure 1D,E). Phenotypic selection has produced individual plants that are nearly nonshattering and mostly free threshing. As the traits are highly polygenic and *T. intermedium* is an outcrossing polyploid, the challenge is to obtain a population that breeds true for the desired traits. This may be achieved in a matter of generations using marker-assisted/genomic selection, but could turn out to be a persistent problem.

Non-Brittle Rachis

Seed shattering in barley (*Hordeum vulgare*) and other grasses is the result of detachment of the spikelet from the spike. The central part of the spike is the rachis, composed of a number of short nodes and internodes. Spikelets grow at the nodes. Wild barley spikes present thin primary and secondary cell walls at rachis nodes, which makes them brittle and prone to shedding the seeds at maturity [35], while domesticated barley varieties have thickened cell walls at rachis nodes. Although the exact mechanism is unknown, this phenotype is related to loss of function of either one of two genes, namely *Btr1* or *Btr2* [35–37], and it was suggested that these genes were significant drivers in the evolution of the rachis-type disarticulation system of Triticeae grasses. In barley, closely related genes named *Btr1-like* and *Btr2-like* have also been identified, but they are not functional paralogs of *Btr1* and *Btr2* as they cannot complement *btr1* and *btr2* in cultivated barley [35].

Spikes of *T. intermedium* have multiple brittle break points. In some genotypes, the break points are above the node, while in others they are below, which suggests that avoiding shattering is a complex problem. The *T. intermedium* genome has several genes that resemble *Btr1* and *Btr1-like* and also *Btr2* and *Btr-2-like* (Table 1). Among the encoded proteins, nine show more than 50% amino acid sequence identity to Btr1 and nine more than 50% identity to Btr2. The hydrophobicity profile of the barley Btr1 protein is distinct from that of barley Btr1-like in that two transmembrane spanning segments appear to be present in Btr1 but absent from Btr1-like [35]. At least the six best hits among putative Btr1 proteins have a hydrophobicity profile that resembles that of barley Btr1. This would suggest that multiple homologs of *Btr1* and possibly also *Btr2* are present in the *T. intermedium* genome. *Btr1* therefore appears to be a challenging target, but mutation of *Btr2* may be sufficient to obtain a reliable non-brittle rachis.

Free Threshing

In cereals like rice (*Oryza sativa*) and wheat, seed shattering results from the detachment of the seed from the stalk connecting it to the glume (leaf-like structures) that tightly enclose the grain.

Table 1. Candidate Domestication Genes That Align with Intermediate Wheatgrass QTLs

Trait	Gene	Gene name	Source	IWG HG[a]	IWG V2.1 gene model	Identity (%)[b]	Refs
Non-brittle rachis	*Btr1*	*Non-brittle rachis1*	Barley (3)	3	Thint.09G0046000 Thint.07G0115700 Thint.07G0115500 Others[c]	87,24 88,27 86,73	[35,82]
Non-brittle rachis	*Btr2*	*Non-brittle rachis2*	Barley (3)	3	Thint.08G0083300 Thint.09G0045900 Thint.07G0116500	84,65 85,64 81,19	[35,82]
Free threshing	*nud*	*Naked*	Barley (7)	7	Thint.21G0474100 Thint.19G0474200 Thint.20G0730900	98,12 90,14 87,85	[38,83]
Free threshing	*Q*	*Spelt Factor (Free Threshing*	Wheat (5A)	5	Thint.13G0534400 Thint.14G0534400	91,92 90,85	[43]
Free threshing	*qSH1*	*QTL of seed shattering chr1*	Rice (1)	3	Thint.09G0471600 Thint.07G0416900 Thint.07G0432000	64,48 72,00 70,05	[39]
Free threshing	*SH4*	*QTL of seed shattering chr4*	Rice (4)	4	Thint.10G0033500 Thint.11G0014200 Thint.12G0385700	48,59 48,25 45,77	[84]
Free threshing	*SH5*	*SH1-like gene on chr5*	Rice (5)	1	Thint.01G0340000 Thint.02G0511800 Thint.03G0500300	71,30 72,12 73,55	[41]
Free threshing	*SHAT1*	*Shattering abortion 1*	Rice (4)	2	Thint.04G0397200 Thint.06G0067900	84,50 84,56	[40]
Grain size	*GASR7*	*Gibberellic Acid-Stimulated Regulator*	Wheat (7)	7	Thint.19G0343500 Thint.20G0544200 Thint.21G0194900	94,95 98,02 93,68	[57]
Grain size	*GW2*	*Grain Width chr2*	Wheat (6D)	6	Thint.16G0144700 Thint.17G0207800 Thint.18G0258700	98,11 97,88 98,59	[60,61,85–87]
Grain size	*GW5 (qSW5)*	*Grain weight QTL chr5*	Rice (5)	1	Thint.02G0278200 Thint.03G0309500 Thint.01G0175600	75,52 74,12 75,10	[59]
Plant height	*RHT*	*Reduced Height*	Wheat (4)	4	Thint.10G0079300 Thint.12G0065200 Thint.V1841900	98,49 94,33 92,73	[51]
Seed set	*GNI1 (Vrs1)*	*Grain Number Increase 1 (Six-Rowed Spike 1)*	Wheat (2), Barley (2)	2	Thint.04G0233800 Thint.05G0279100 Thint.05G0085200 Thint.06G0293200 Thint.06G0432200	87,27 66,27 86,76 85,52 83,33	[64,88]
Spike density	*Zeo*	*Zeocriton*	Barley (2)	2(7)	Thint.04G0397200 Thint.06G0067900 Thint.21G0065000	87,47 84,79 84,64	[52]

[a]Abbreviations: HG, homoelogous group; IWG, intermediate wheatgrass.
[b]Percent identity of predicted amino acid sequences.
[c]Six physically close genes have a sequence identity of >50%: Thint.07G0115400, Thint.07G0116600, Thint.07G0116000, Thint.08G0083500, Thint.09G0044900, and Thint.09G0045800.

Deletions and single nucleotide polymorphism (SNP) mutations of the barley *nud* gene are responsible for the free-threshing trait in barley [38]. In rice, transcription factors such as *qSH1* (Os01g62920) [39] and *SHAT1* [40] are associated with defects in the formation of the abscission layer that promotes seed detachment. Suppressed expression of a *qSH1* homolog, *SH5* [41], results in reduced seed shattering. While *qSH1* and *SH5* are homeodomain transcription factors,

SHAT1 belongs to the *Arabidopsis thaliana APETALA2* family, whose members seem to be important for domestication in other grasses.

In wheat, in particular, one member of this family, the *Q* gene, is known for its role in the domestication of this grass. *Q* is the result of mutation(s) in *q*. As a consequence, the elongated spike becomes subcompact and the glumes fragile, which result in free-threshing grains [42]. Two SNPs have been hypothesized to be responsible for the phenotype of *Q*. One SNP causes an amino acid change (Ile329Val) in the predicted Q protein with the functional consequence that the protein has difficulties forming homodimers, which are required for its proper function [43]. The other SNP does not alter the coding sequence, but changes the binding site for a miRNA (miR172) that regulates the expression of *Q* [44–46]. Modifying this miRNA target is sufficient to produce the phenotype of *Q*, suggesting it is the more important of the two SNPs [47]. Loss-of-function mutations of the corresponding gene in other plants result in more drastic phenotypic changes, such as defects in flower development, which is why the *Q* phenotype reflects more subtle changes in the regulation of the gene.

Genetic studies in *T. intermedium* led to the identification of quantitative trait loci (QTLs) for free threshing and other traits [48], but so far have not led to the identification of a causative gene for free-threshing grains. There are three putative orthologs of the barley *nud* gene on LG19, LG20, and LG21 (**homoeologous group** 7; Thintv21282433, Thintv21312339, and Thintv21331531, respectively) of *T. intermedium* (Table 1). The *T. intermedium* predicted proteome includes two homologs of wheat Q (Thint.14G0534400 and Thint.13G0534400), both having very high amino acid sequence identity to Q (91% and 92%, respectively) (Table 1). Compared with Thint.14G0534400, Thint.13G0534400 lacks the 72 N terminal residues and may therefore represent a pseudogene. Ile-329 in ancestral wheat corresponds to Ile-325 in Thint.14G0534400 and is encoded by a GTC codon. To mutate this codon into a codon encoding Val, a G-to-A mutation would be required. It would be extremely challenging to identify such a specific substitution in a mutant population of *T. intermedium* using TILLING approaches; however, it should be feasible with the use of cytosine-**base editors** that convert C to T, and a G-to-A mutation can be easily achieved in the complementary DNA strand [49,50]. Further downstream in the sequence, the miR172 target site (5′-CT GCA TCA GGA TTC T-3′) is 100% conserved between ancestral wheat and *T. intermedium*. The SNP in *Q* is a C-to-T conversion at the penultimate position in this sequence. TILLING approaches to identify such a mutation would likewise pose a formidable challenge, but can be accomplished by use of cytosine base editors [49,50].

Plant Height

The taller the plant, the greater the risk that bad weather conditions will cause lodging and yield losses. In wheat, the semi-dominant wheat *Reduced Height-1* genes (*Rht-B1* and *Rht-D1*) are known as the ‘green revolution genes’ and their variants have produced marked increases in yield of wheat (and rice) since their introduction into widely used cultivars in the 1960s [51]. The *Rht-B1* and *Rht-D1* mutations reduce plant height by reducing the response to gibberellin, but null mutations actually increase plant height. These are not simple knockout mutations, but there are several documented dwarf mutations from *Arabidopsis*, maize (*Zea mays*), and wheat that provide patterns that can be followed.

In the *T. intermedium* genome, two putative *rht* orthologs on LG10 and LG12 (homoeologous group 4; Thintv21141900 and Thintv21164166, respectively) are present (Table 1). The putative *rht* ortholog on LG10 aligns with the strongest plant height QTL in the Larson *et al.* [48] study and is a promising target for gene editing.

Spike Density

Another trait related to plant height is spike density. The *Zeocriton* (*Zeo*) gene on barley chromosome 4 regulates spike density. Houston *et al.* [52] identified SNPs in the microRNA172 binding site of the barley *Zeo* gene. The stability of the *Zeo* transcript is regulated by microRNA172-mediated cleavage, with reduced cleavage resulting in shorter internodes.

In *T. intermedium*, three putative *Zeo* orthologs are present on LG4, LG6, and LG21 (Thintv21058456, Thintv21074524, and Thintv21321694, respectively; Table 1) and all contain the same 'wild type' microRNA172 binding site sequence. These genes are possible targets of gene editing through disruption of the microRNA172 binding sites.

Other traits have turned out to be extraordinarily challenging to breed for, such as increasing seed size/plumpness and seed set. Obtaining highly fertile variants with plump seeds could be transformative.

Grain Size

In contrast to the genes underlying the seed shattering phenotype, most domestication genes controlling grain size are not transcription factors [53,54]. Grain length has been associated with the *GASR7* gene. *GASR7* genes are mostly hormone-regulated and encode proteins of the Snakin/GASA family involved in signaling pathways that modulate hormonal responses [55]. *TuGASR7* is associated with increased grain length and weight in the parental wheat species *Triticum urartu* [56] and *TaGASR7* is a major genetic determinant of grain length in hexaploid wheat [57,58].

In rice, a QTL for grain width has been mapped to a gene on chromosome 5 (*GW5*), immediately downstream of a region where the Nipponbare genetic background (wide grains) presents a chromosomal deletion of 1.1 kb with respect to the Kasalath cultivar (thin grains) [59]. *GW5* encodes a calmodulin binding protein that mediates brassinosteroid (BR)-responsive gene expression and growth responses, including grain size [59]. Another QTL for increased grain width in rice is the *GW2* gene. This gene encodes a type E3 ubiquitin ligase that negatively regulates grain width by controlling the number of cells in the spikelet hulls [60]. The *OsGW2* QTL has also been shown to contribute to increased grain size by affecting the milk filling rate of the grain [60]. Common wheat contains three homologs of *GW2*. Loss-of-function mutations in all three genes simultaneously induced by CRISPR-Cas9 technology resulted in wider and longer grains and hence increased yield [61].

In the *T. intermedium* genome, three genes (Thint.19G0343500, Thint.21G0194900, and Thint.20G0544200) encode proteins with a very high level of amino acid sequence identity (between 91% and 95%) to GASR7 in hexaploid wheat (Table 1). Likewise, three genes in the *T. intermedium* genome (Thint.18G0258700, Thint.16G0144700, and Thint.17G0207800) (Table 1) encode proteins closely related to wheat GW2. The amino acid identity to wheat GW2 is extremely high (97–98%). The high identity of *T. intermedium*-predicted proteins to GASR7 and GW2 suggests that they are functional homologs of these proteins. Currently, *T. intermedium* seeds are already as long as wheat seeds, but very thin (Figure 1E,F), and even longer seeds are more likely to break in harvest and processing. Work on the *GW2* gene or other genes (such as *GW5*) related to plumpness should therefore be a priority. As loss-of-function mutations are sufficient to produce the domestication phenotypes, both TILLING and genome editing approaches should be feasible to obtain mutant plants in which all six alleles of both *GASR7* and *GW2* have been neutralized.

Seed Set

Fertility determines the number of grains per spike and is a major determinant of yield. Currently, the best seed set rates in *T. intermedium* are as low as 30% and increasing seed set would be a

rapid way to boost yield. Information concerning the genetic basis for fertility and seed set has recently become available for wheat and barley [62,63]. The *GNI1* gene encodes an HD-Zip I transcription factor and its mutations in wheat and barley have led to increased floret fertility, higher grain number per spike, and higher yields [63,64]. Wheat GNI-A1 (SPT19245) has been under selection during domestication and its reduced function in domesticated wheat is the result of an Asn105Tyr mutation in the otherwise conserved HD domain. Two homologs of wheat SPT19245 are present in *T. intermedium*: Thint.06G0432200 and Thint.05G0279100 (Table 1). One of these (Thint.05G0279100) is most likely a pseudogene as it has an internal deletion in the conserved HD domain. A related gene of SPT17206 is present in the wheat genome, with three homologs present in the *T. intermedium* genome. To make the corresponding Asn-to-Tyr mutation in Thint.06G0432200, an AAC codon must be mutated to TAC. Currently, no base editor tested in plants can generate an A-to-T mutation, but a new genome editing strategy called 'prime editing', which can accomplish all 12 types of point mutations, has been developed in human cells [65] and similar strategies in plants may be anticipated in the near future.

Strategies for Targeted Mutagenesis

Few genetic transformation protocols and no genome editing studies have been reported for *T. intermedium* [66]. To increase the gene transformation efficiency, both *Agrobacterium*-mediated and biolistic transformation methods need to be optimized and established in *T. intermedium*. Since the limited genetic modification in *T. intermedium* could be attributed to its recalcitrance to adventitious shoot regeneration in tissue culture [67], one of the most efficient ways to stimulate the regeneration in monocot plants is the heterologous expression of booster genes such as maize *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) [68]. The maize *Bbm* and *Wus2* genes have been successfully used to stimulate transformation of maize immature embryos, sorghum (*Sorghum bicolor*) immature embryos, sugarcane (*Saccharum officinarum*) callus, and indica rice (*Oryza sativa* ssp. *indica*) callus [68].

Stable transformation, however, is not a requirement for genome editing. Editing also occurs when DNA encoding Cas9 and single-guide RNA is delivered into plant cells without integration and, indeed, the mutagenesis frequency of transient expression-based gene editing techniques is higher than that of conventional, integration-based genome editing methods [58]. In 2016 and 2017, using particle bombardment to deliver CRISPR/Cas ribonucleoproteins (RNPs), two groups developed DNA-free genome editing tools in wheat and maize, respectively [69,70], and Kim *et al*. used CRISPR/Cas12a RNPs to genetically modify tobacco and soybean protoplasts [71]. Recently, it was shown that **base editing** APOBEC3A (A3A) deaminase-RNPs could alter targeted base pairs in wheat [50]. Consistently, DNA-free editing not only produced edited plants that lacked any trace of foreign DNA, but also improved editing specificity.

Tissue culture is a time-consuming and laborious process. To sidestep the need for tissue culture in wheatgrass genome editing, alternative strategies for delivering gene editing reagents are being developed. First, an RNA virus-based delivery system in which the gRNA is delivered into plants by viruses has been reported [72,73]. Second, carbon nanotubes and the mesoporous silica nanoparticles can deliver DNA into mature plant tissues [74,75]. Third, developmental regulators and gene editing reagents can be co-delivered into somatic cells of intact plants, which are further induced to produce meristems that develop into gene-edited shoots [76]. In this method, developmental regulators and gene editing reagents are co-delivered into somatic cells, which are further induced to produce meristems that develop into gene-edited shoots.

If targeted mutagenesis cannot readily be achieved in *T. intermedium*, an alternative approach would be needed to identify useful genetic variants present in wild accessions. As an outcrossing

polyploid species, *T. intermedium* contains abundant genetic variation, much of which is in the form of 'genetic load', or nonfunctional allelic forms [24]. Most of these alleles are expected to be at least partially recessive and therefore may not produce novel phenotypes so long as dominant homoeologous alleles are present in one of the genomes. Therefore, potentially useful genetic variants might only be discovered by sequencing target alleles in diverse material. This approach has been outlined for the rapid domestication of trees and has been described as 'next-generation EcoTILLING' [77].

Obtaining domestication alleles through editing, transformation, or discovery in a wild relative is only the first step towards developing useful crop varieties. If more than one novel mutation is required to obtain the target phenotype, each of these unique alleles will have to be introduced into breeding populations. Intermatings and selection must proceed in such a manner as to fix these new alleles in the population (where 100% of plants now carry the new alleles). Theoretical strategies to expedite this process have been developed [78] and marker-assisted backcross breeding has been used to successfully pyramid numerous unique genes [79]. In an outcrossing species, genetic markers can be used to eliminate diversity at a particular locus while maintaining diversity across the genome in the breeding population [80], but numerous generations will be required.

Outstanding Questions

Are loss-of-function mutations and/or weakening mutations sufficient to domesticate a perennial grass?

Can a perennial grass be domesticated solely by mutating only a handful of genes?

If domestication of *T. intermedium* is achievable by mutagenesis approaches, would the principle then apply to other perennial grasses as well?

Can a perennial grain crop be developed that has the same yields as annual grain crops?

Concluding Remarks

Key domestication genes in barley, wheat, and rice are also present in the *T. intermedium* genome. Some (like *Btr1*) present challenging targets as they are present in many copies and it is uncertain whether all are functional homologs of domestication genes. Others, like GASR7 and GW2, are present in three copies only, similar to what is seen in wheat, and the encoded proteins share such a high level of amino acid sequence identity to their wheat counterparts that they are likely functional homologs. Knockouts of these genes in *T. intermedium* could possibly cause domestication phenotypes. Knockouts in individual alleles could be identified by TILLING approaches and would have to be followed by crosses of different knockout lines to obtain a homozygous population containing mutant alleles at all loci. Alternatively, multiplex genome editing technologies could be employed to knock out all six alleles simultaneously [81]. This approach is currently limited by the lack of a transformation protocol for *T. intermedium*. Finally, a single copy of a gene resembling *q*, an ancestor of the major domestication gene *Q* of wheat, was identified in the *T. intermedium* genome. To convert this gene into a *Q* homolog, single nucleotide substitutions are required. Whereas these substitutions cannot easily be generated using TILLING approaches, they should be achievable using current methods for base editing, provided *T. intermedium* can be transformed (see Outstanding Questions).

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