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The pseudogenes of barley

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SUMMARY

Pseudogenes have a reputation of being 'evolutionary relics' or 'junk DNA'. While they are well characterized in mammals, studies in more complex plant genomes have so far been hampered by the absence of reference genome sequences. Barley is one of the economically most important cereals and has a genome size of 5.1 Gb. With the first high-quality genome reference assembly available for a Triticeae crop, we conducted a whole-genome assessment of pseudogenes on the barley genome. We identified, characterized and classified 89 440 gene fragments and pseudogenes scattered along the chromosomes, with occasional hotspots and higher densities at the chromosome ends. Full-length pseudogenes (11 015) have preferentially retained their exon–intron structure. Retrotransposition of processed mRNAs only plays a marginal role in their creation. However, the distribution of retroposed pseudogenes reflects the Rabl configuration of barley chromosomes and thus hints at founding mechanisms. While parent genes related to the defense-response were found to be under-represented in cultivated barley, we detected several defense-related pseudogenes in wild barley accessions. The percentage of transcriptionally active pseudogenes is 7.2%, and these may potentially adopt new regulatory roles.The barley genome is rich in pseudogenes and small gene fragments mainly located towards chromosome tips or as tandemly repeated units. Our results indicate non-random duplication and pseudogenization preferences and improve our understanding of the dynamics of gene birth and death in large plant genomes and the mechanisms that lead to evolutionary innovations.

Keywords: pseudogenes, barley, *Hordeum vulgare*, plants, gene fragments, gene evolution.

BACKGROUND

Pseudogenes are generally referred to as 'evolutionary relics' or 'junk DNA'. They are genomic sequences similar to functional genes but which contain degenerative features such as mutations like frameshifts or premature stop codons, leading to a loss of their original function. Consequently, pseudogenes have been disregarded in routine plant genome annotations and continuative studies.

Most pseudogenes originate from a duplication event. The functional counterpart is termed a 'parent' gene (Tutar, 2012). If the gene copy did not become defective immediately after its duplication, genetic redundancy will lead to a

relaxed selection pressure and degeneration of one of the copies is tolerated. For scenarios in which one copy becomes defective, a gene–pseudogene pair arises (Balakirev and Ayala, 2003).

Gene duplication can be triggered by different cellular mechanisms (Podlaha and Zhang, 2010). Unequal crossing-over during meiosis can lead to tandem duplications. If sister chromatids are not separated properly during cell division (non-disjunction), chromosome duplications are the result. The duplication of whole genomes, polyploidization, is particularly widespread among plants (Weiss-Schneeweiss *et al.*, 2013). Pseudogenes originating from any of these mechanisms are termed 'duplicated' or 'non-processed' (Podlaha and Zhang, 2010). Alternatively, duplication can occur via a mRNA intermediate and re-insertion of reverse-transcribed cDNA into the genome. These 'retroposed' or 'processed' pseudogenes are considered as 'dead-on-arrival', because they lose their upstream promoter and regulatory sequences during duplication. Processed pseudogenes are characterized by a loss of intron sequences, poly-A tails near the 3′ ends and small flanking direct repeats (Sen and Ghosh, 2013). Unitary pseudogenes comprise the third type of pseudogene (Zhang *et al.*, 2010). These are thought to arise rarely and without prior gene duplication. In humans, olfactory receptor genes (387) form one of the largest gene families, which has numerous pseudogenes (415). It is hypothesized that the development of color vision reduced the importance of odor sensing and resulted in the pseudogenization of numerous olfactory receptor genes (Vihinen, 2014).

In recent years, gene look-alikes have attracted particular attention because of reported cases of pseudogene functionality (Pink *et al.*, 2011; Sen and Ghosh, 2013). Despite their lost protein-coding potential, some are still transcribed and able to play a role in regulatory processes (Balakirev and Ayala, 2003; Poliseno *et al.*, 2010). Due to the sequence similarity to bona fide genes, their transcripts can interfere with the translational machinery or be used for gene regulation via small interfering RNA (siRNA) or microRNA (miRNA) synthesis (Pink *et al.*, 2011). Pseudogenes are now increasingly being studied in mammals. For instance, human pseudogenes are of particular interest in the context of diseases (Pink *et al.*, 2011; Roberts and Morris, 2013; Sen and Ghosh, 2013). Their altered expression has been linked to cancer, where they can now be used as markers for specific cell types (Poliseno *et al.*, 2015). In contrast, for most plant species, no genome-wide pseudogene annotations are yet available. Until recently, pseudogene studies in more complex plant genomes such as the Triticeae (e.g. wheat, barley, rye) were hampered by the absence of high-quality assembled reference genome sequences. Cultivated barley (*Hordeum vulgare L.*) is one of the five economically most important cereal species and a member of the Triticeae tribe (Spannagl *et al.*, 2013). Its diploid genome has a size of 5.1 gigabases (Gb) – making it 2 Gb larger than the human genome – and comprises 39 734 high-confidence gene loci (Mascher *et al.*, 2017). Sequencing and genome assembly efforts have been hampered by its highly repetitive genome: about 80% consists of transposable elements. With one of the first true reference genome assemblies now being available for a Triticeae crop and the first bacterial artificial chromosome (BAC)-by-BAC assembly of a genome of such size (Mascher *et al.*, 2017), we conducted a genome-wide assessment of potential pseudogenes in barley. We exploited the homology of pseudogenes to their parent genes to identify them and then classified them into duplicated or retroposed pseudogenes. We studied their distribution along the chromosomes, their relation to genes and gene families and their functional potential. Then we analyzed syntenic regions between cultivated barley (cv. Morex) and four wild barley accessions (Tan *et al.*, 2017) and investigated pseudogene differences. Our results enable a deeper understanding of pseudogenes in cultivated and wild crops and provide the basis for detailed analyses of potentially functional pseudogenes. Novel insights into the mechanisms underlying the genesis of pseudogenes, and thus a major evolutionary force underlying genome evolution, are generated. Pseudogenes are a 'playground for innovation', since their usual non-functionality allows them to accumulate mutations without effects on fitness. However, their gene-like structure improves their potential for subsequent resurrection and adoption of novel functional roles.

RESULTS

Pseudogenes and gene fragments

The barley genome contains a vast number of gene fragments and pseudogenes. Using a homology-based approach (Figure S1 in the Supporting Information), we identified 89 440 potential pseudogenes, most of which constitute short gene fragments with an average coding sequence (CDS) length of only 188 base pairs (Table 1, Figure 1). In comparison, protein-coding genes have an average CDS length of roughly 1 kb (Table S1). Similar large quantities of short gene fragments have been found in the genome of hexaploid wheat (Brenchley *et al.*, 2012). In barley, 12.3% (11 015) of the pseudogenes represent full-length copies of genes (Table 1). Those 'traditional' pseudogenes cover the CDS of their parent gene by at least 80% and are called high-coverage (HC) pseudogenes hereafter.

The chromosomal and genomic distribution of pseudogenes largely remodels the distribution found for functional genes and gives a mirror image of transposable elements (Figure 2). We observed that some parent genes have a particularly large number of pseudogene homologues. As in wheat (Brenchley *et al.*, 2012), many of those

Table 1 Basic metrics for all pseudogenes and high-coverage (HC) pseudogenes found in the barley genome

Pseudogene class	Number	%	Mean length (bp)	Mean coverage (%)	Mean identity (%)
All					
All pseudogenes	89 440		188	33.5	91.4
Duplicated	12 556	14.0	329	40.7	93.8
Processed	1834	2.1	238	29.3	91.4
Chimeric	571	0.6	423	35.5	93.4
Single exon parent	38 424	43.0	190	46.4	90.3
Fragmented	36 055	40.3	130	17.4	91.7
HC					
All pseudogenes	11 015		376	94.6	93.0
Duplicated	2151	19.5	540	95.1	95.1
Processed	153	1.4	509	93.6	90.1
Chimeric	41	0.4	713	90.7	94.2
Single exon parent	8224	74.7	329	94.8	92.5
Fragmented	446	4.1	378	89.8	93.3

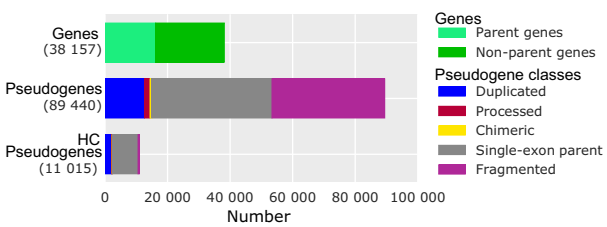


Figure 1. Gene and pseudogene metrics.
The number of genes and parent genes, as well as pseudogene classes for all and high-coverage (HC) pseudogenes, respectively.

fragments may actually be common domains found multiplied in the genome (Figure 2). Nevertheless, 1560 pseudogenes are highly similar to their parent gene in both length and sequence identity (≥98% similarity). These gene facsimiles are well represented in among duplicated pseudogenes (Figure 3b), but to a smaller degree in retroposed pseudogenes (Figure 3c). This is consistent with the hypothesis that retroposed pseudogenes accumulate mutations immediately (dead-on-arrival) and thus diverge faster from their parent genes than duplicated pseudogenes (Thibaud-Nissen *et al.*, 2009).

Retroposed pseudogenes are copies resulting from reinsertion of reverse-transcribed mRNA into the genome. Unlike duplicated pseudogenes, they lose their introns during the maturation of mRNA. Of the full-length HC pseudogenes in barley, 2151 contain introns at corresponding parent splice sites and can be classified as duplicated pseudogenes (Table 1). In contrast, only 153 HC pseudogenes appear to originate from retrotransposition. The remainders are pseudogenes that cannot be classified into duplicated or retroposed based on their exon–intron structure. They are either too short to cover intron junctions (fragmented), are chimeric or their parent gene only comprises a single exon (Figure S1).

Distribution on chromosomes

Duplicated pseudogenes most often arise from unequal crossing-over during meiosis, segmental duplications or chromosome duplications and polyploidization events (Podlaha and Zhang, 2010). Most plants have a long evolutionary history of duplications and chromosome rearrangements (Gaut *et al.*, 2000; Yu *et al.*, 2005; Bolot *et al.*, 2009; Heslop-Harrison and Schwarzacher, 2011). With a large number of pseudogenes found to be duplicated, we analyzed whether these are located in close vicinity to their parent gene, and are thus likely the result of unequal crossing-over events, or if they are more randomly distributed across the chromosomes, as expected for retroposed pseudogenes and segmental duplications derived by other mechanisms than unequal crossing-over (Figure 4). Of all HC pseudogenes, 3.1% are located within 50 kb of their respective parent gene. As expected, a significantly larger portion of HC pseudogenes classified as duplicated were found to be located within this close range to their parent gene (4.8%; binomial test, *P*-value 2.2×10^{-5}) (Table S1). Also, pseudogenes with a higher sequence similarity to their respective parent genes are likely to have a younger divergence time or are affected by gene conversion. Tandem duplicated pseudogenes are preferentially affected by gene conversion events with their parents that potentially decelerate the sequence divergence between the pair. Indeed, we found pseudogenes in close vicinity to their parent genes to be more similar to them (Figure S2). However, this does not just apply to duplicated pseudogenes but also to retroposed pseudogenes, and is indicative of gene conversions and sequence homogenization events independent of the duplication mechanism.

Moreover, not only duplicated but also retroposed pseudogenes were found to be preferentially located on the same chromosome as their parent gene (20.6%;

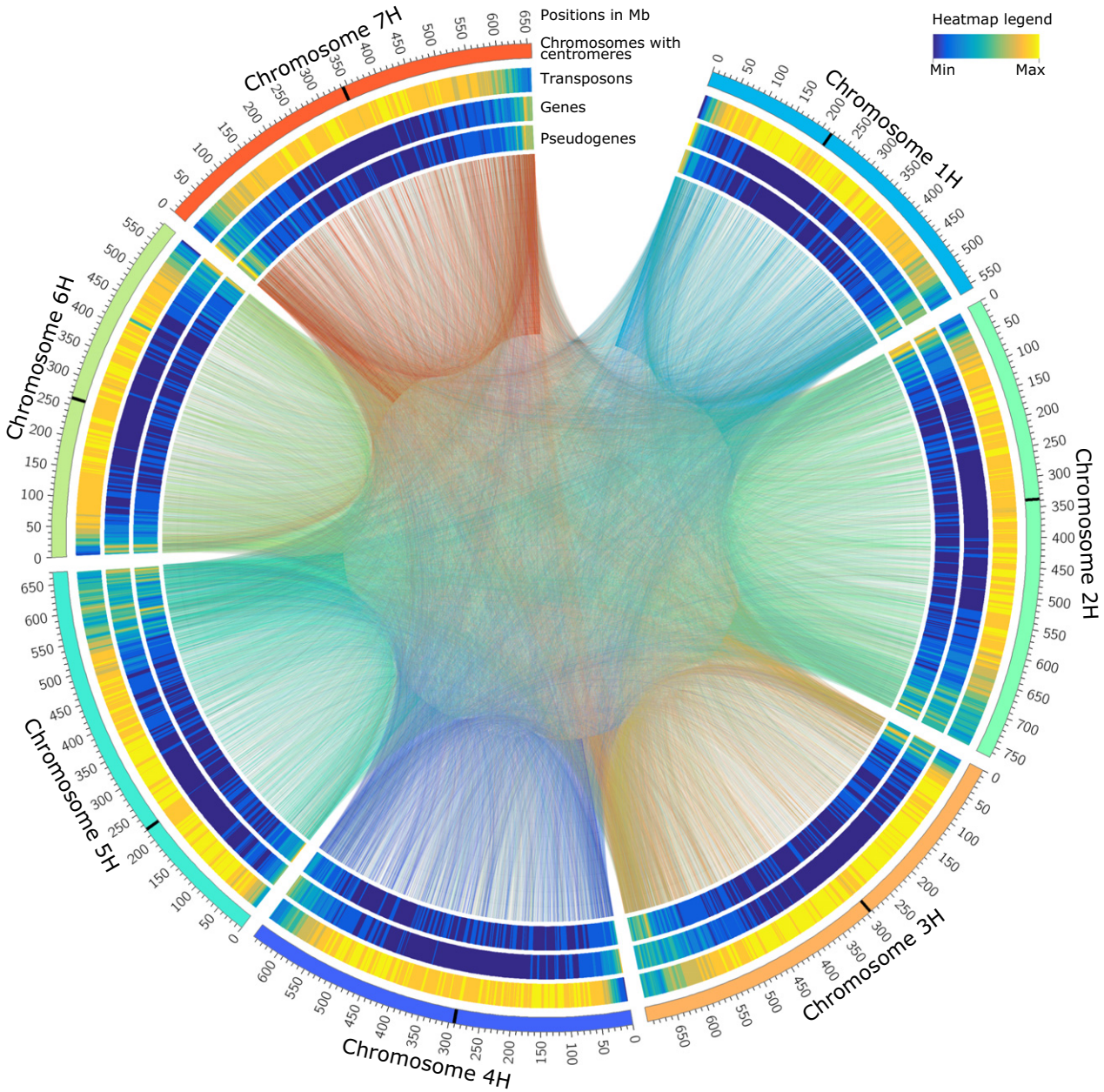


Figure 2. Distribution of transposable elements, genes and pseudogenes on the seven chromosomes of barley.
The first (outer) track shows the seven barley chromosomes with positions in Mb and highlighted centromeres. The second to fourth tracks show densities of transposable element sequences (minimum 47% to maximum 85% sequence coverage), genes (minimum 0% to maximum 5% sequence coverage) and pseudogenes (minimum 0% to maximum 2% sequence coverage), respectively. Densities have been calculated using a sliding window of 5 Mb shifted by 1 Mb. Links in the center connect parent genes with their pseudogene 'descendants' and are colored in the chromosome of the respective parent gene. Tandem duplicates can be easily recognized as straight lines, in particular at the chromosome ends.

chi-square test, *P*-value 2.4×10^{-4}). This contradicts the assumed random reinsertion of reverse-transcribed cDNA during retrotransposition. A preferential reinsertion of the cDNA on the same chromosome, or even in the vicinity of its origin, is unlikely for a long terminal repeat (LTR) retrotransposon-mediated transfer, for which the reverse transcription takes place in the cytosol. However, the presence of retroposed pseudogenes at a significantly higher rate locally or on opposing chromosome arms may be explained by an alternative scenario. As in humans, non-LTR-retrotransposons (LINEs) are likely to carry out reverse transcription directly at the integration site in the nucleus (Esnault *et al.*, 2000; Kaessmann *et al.*, 2009). In humans, the ORF1p protein of LINE L1 has been

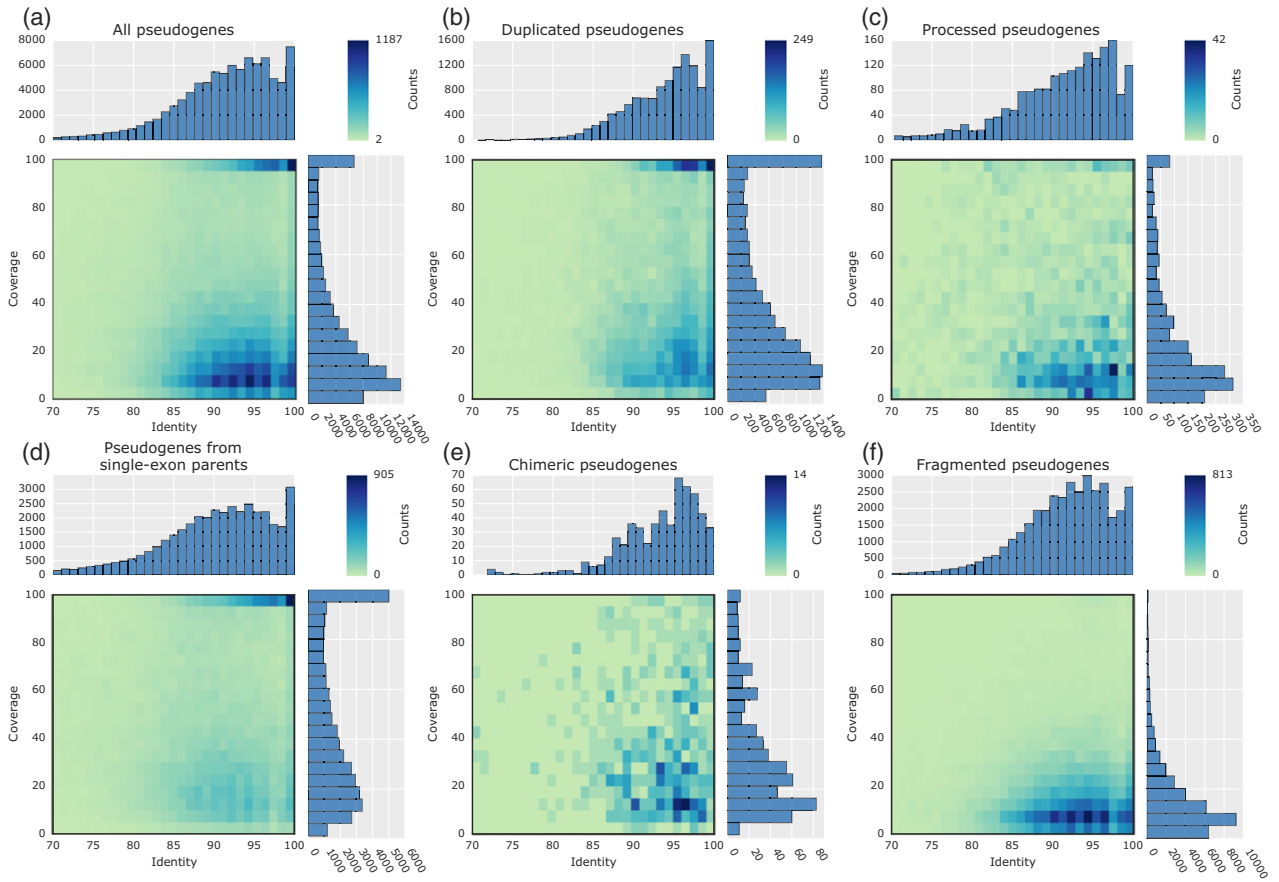


Figure 3. Sequence coverage versus identity of barley pseudogenes and their subclasses compared to their respective parent genes.
(a) All pseudogenes, (b) duplicated pseudogenes, (c) processed pseudogenes, (d) pseudogenes from single-exon parent genes, (e) chimeric pseudogenes, and (f) fragmented pseudogenes.

shown to bind cellular mRNA, which can serve as a template for reverse transcription (Mandal *et al.*, 2013). The colocalization of transcription and LINE-driven reverse transcription might thus lead to a preferential retrotransposition in physical proximity to the transcribed parent gene. The barley genome contains 7780 LINE elements within 10 kb of one of the 28 316 high-confidence genes (Wicker *et al.*, 2017) – out of the 19 173 LINE elements in the genome in total (Mascher *et al.*, 2017). Compared with the chromosomes of many other eukaryotes, individual barley chromosomes fold back to juxtapose the long and short arms (Mascher *et al.*, 2017). This so-called Rabl configuration is adopted in interphase nuclei and leads to reduced distances between corresponding chromosome arms (Dong and Jiang, 1998). The Rabl configuration thus might increase the probability of insertion of retroposed pseudogenes on the same chromosome as the parent gene. Indeed, we found many intrachromosomally retroposed pseudogenes to be located either close to their parent gene or on the opposing chromosome arm (Figure S3).

Tandem gene clusters and larger gene families are birthplaces of pseudogenes

Manual inspection of barley pseudogenes in the Integrative Genomics Viewer (IGV) (Thorvaldsdóttir *et al.*, 2013) hinted towards pseudogene hot-spots at tandem gene clusters. To statistically confirm this, we assessed the proportion of pseudogenes in close vicinity to tandem gene clusters. Considering all HC parent genes, 8.7% of them are located in close vicinity to at least one of their HC pseudogene 'descendants'. However, if we focus only on HC parent genes located within a tandem gene cluster, we find a significantly increased proportion (37.8%; binomial test, *P*-value 2.2×10^{-16}) to be close to a HC pseudogene 'descendant'. The observed four-fold relative difference supports the hypothesis of accumulation of pseudogenes in tandem gene clusters.

Additionally, we confirmed a positive correlation between gene family size and HC pseudogene content (Figure 5a). Not surprisingly, larger gene families are more likely to give rise to pseudogenes (Zou *et al.*, 2009), since

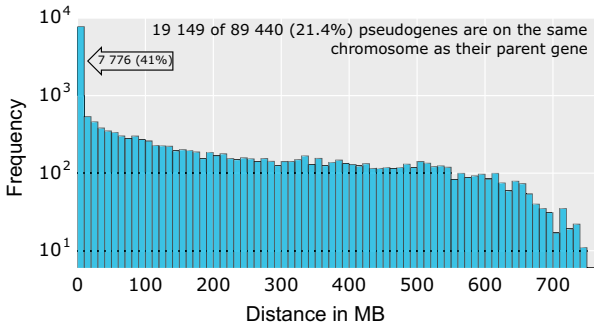


Figure 4. Distance distribution of pseudogenes to their respective parent genes.

expansion gives the opportunity to evolve new functionalities but also to balance eventual pseudogenization of individual gene family members. To study how the pseudogene content changes during the expansion or contraction of a gene family we compared orthologous groups of barley with *Arabidopsis thaliana*, *Brachypodium distachyon*, rice and sorghum (Figure 5b). Barley contains 1954 expanded and 117 contracted orthologous groups (Mascher *et al.*, 2017). The relative number of pseudogenes per gene family is higher for expanded orthologous groups than for contracted orthologous groups. Consequently, gene duplications leading to an expansion of gene families go hand in hand with the creation of pseudogenes. Respectively, the contraction of gene families does not lead to a high number of pseudogenes – either the genes have degenerated beyond recognition or their sequence has been deleted entirely.

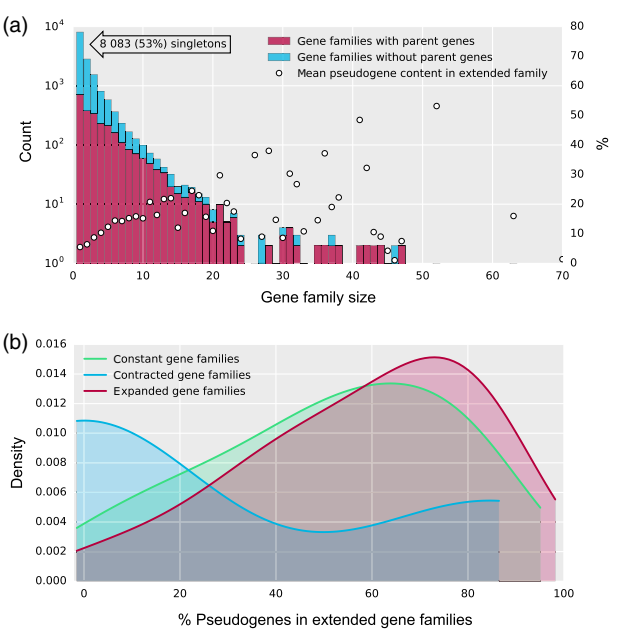


Figure 5. Gene families and pseudogenes.
(a) Relationship of gene family size to high-coverage (HC) pseudogene number. The histogram depicts frequencies of gene family sizes with and without parent gene members (left axis). The dot plot shows the HC pseudogene content in 'extended' families, which are gene families combined with their HC pseudogenes (right axis).
(b) Pseudogene content in 'extended' gene families, that are expanded, contracted or constant in barley compared with rice, sorghum, *Brachypodium distachyon* or *Arabidopsis thaliana*. Only orthologous groups with a minimum size of five were used for this analysis.

Are all pseudogenes non-functional?

Even if degenerate and transcriptionally inactive pseudogenes may still serve as a repertoire of gene-like sequences with the 'capacity to shape an organism during evolution' (Brosius and Gould, 1992). Since it is difficult to prove dysfunctionality – a dogmatic key feature of pseudogenes – there have been several reported cases of pseudogenes which turned out to exert functions (Pink *et al.*, 2011; Sen and Ghosh, 2013). To examine the functional potential and background of barley pseudogenes, we first analyzed the functional annotation of the parent gene set and undertook an enrichment analysis (Figure 6). We found that genes involved in transport, pollination or protein processing are over-represented in the parent gene set. In contrast, genes involved in defense response, stress responses, cell wall organization or sexual reproduction give rise to fewer pseudogenes in barley cv. Morex.

Transcribed pseudogenes have the potential to contribute to the regulation of their parent genes (Pink *et al.*, 2011; Sen and Ghosh, 2013). However, their sequence similarity hampers transcriptional analysis using RNA sequencing (RNA-seq) data, since reads can map ambiguously to both pseudogenes and functional genes. We therefore used only reads mapping uniquely onto the pseudogene sequences. We found transcription evidence for 6435 (7.2%) pseudogenes, 1243 (11.3%) of them from the HC pseudogene set (Table S1). This result is likely to be an underestimation due to the unique mapping of the RNA-seq data. In comparison, about 20% of the annotated pseudogenes in *A. thaliana* and rice are reported to be actively transcribed (Podlaha and Zhang, 2010). Many of the transcribed pseudogenes in barley originate from genes involved in glycolysis or glucose metabolic processes (Figure S4). While evidence for transcription does not necessarily imply functionality, it can nevertheless highlight pseudogenes with regulatory potential.

Selective pressure

We applied a K_a/K_s analysis as an indicator for selective pressure on homologous gene pairs. Pseudogenes are usually under neutral evolution (Podlaha and Zhang, 2010), and we expected a balanced rate of synonymous and non-synonymous substitutions between pseudogenes and their parent genes. Instead, we obtained a K_a/K_s ratio distribution that was significantly shifted to the left, usually seen as indicative of conservation pressure (Figure S5a).

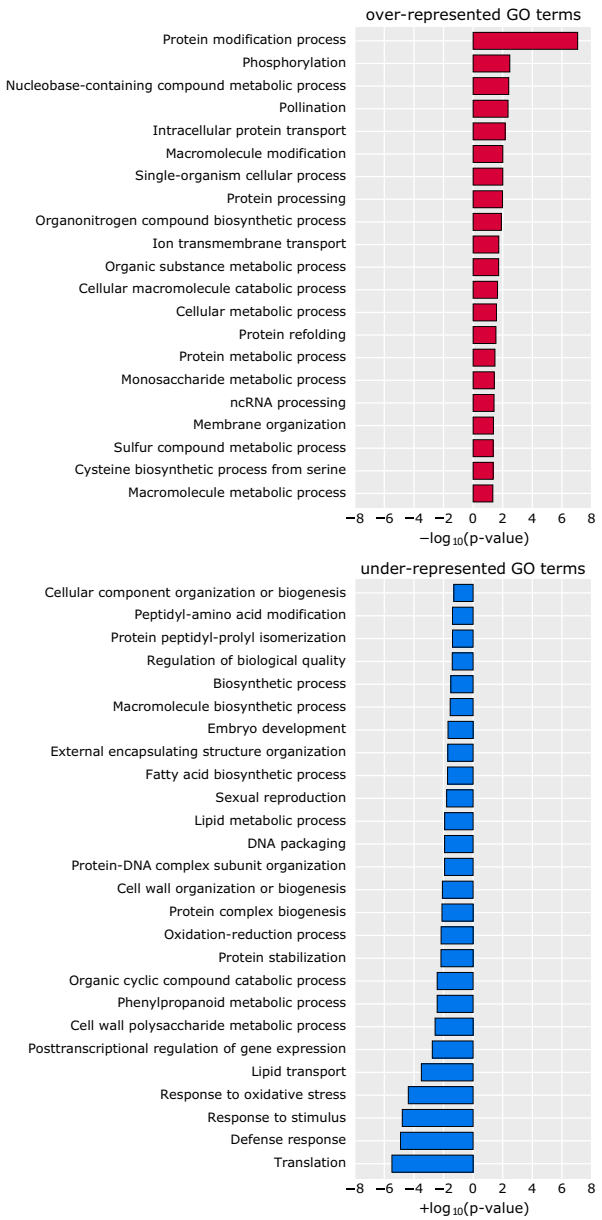


Figure 6. Over- and under-represented Gene Ontology (GO) terms for the parent gene set compared with the complete high-confidence gene set of the barley genome. The sub-ontology 'biological process' was used for this analysis.

Thibaud-Nissen *et al.* (2009) reported similar findings for rice and also gave a convincing explanation (Figure S5b): the parent gene sequences found do not necessarily reflect the parental genes at the time of duplication. Genes accumulate primarily synonymous substitutions, while pseudogenes are expected to accumulate random mutations. If the present parent gene is compared with the pseudogene sequence, a K_a/K_s ratio below 1 is obtained (Thibaud-Nissen *et al.*, 2009).

Duplicated functional genes with defects

Gene duplication is a genomic process that creates new genes and functionalities via neo- and subfunctionalization. In most cases, however, it leads to pseudogenization (Kondrashov *et al.*, 2002; Ho-Huu *et al.*, 2012; Xiao *et al.*, 2016). We identified 4100 (10.8%) functional gene duplicates with a shortened CDS, 255 of which exhibit premature termination codons but are otherwise highly similar to the original version. This result illustrates the other side of the coin: if pseudogenes are interpreted as the byproduct of a mechanism that generates new genes, those 4100 shortened genes can be interpreted as evidence for the generation of new functional genes. Similar to the interpretations by Brosius and Gould (1992), this also confirms the role of pseudogenes as a reservoir of potential genes. Subfunctionalization, neofunctionalization and also pseudogene resurrection are possible outcomes of gene duplication events and drivers in the genetic evolution of genomes.

Comparing syntenic regions between cultivated and wild barley genotypes

Our pseudogene annotation provides the necessary background for detailed analyses of pseudogene evolution, of their impact on genome structure and dynamics and of their potential to interfere with gene regulation. To investigate their evolution in barley subspecies and cultivars, we screened syntenic regions between cultivated barley cv. Morex and four wild barley lines for differences in gene and pseudogene content. Two of the additional genome assemblies are from wild barley accessions growing on opposing slopes of 'Evolution Canyon' I in Israel. The north- and south-facing slopes (NFS and SFS, respectively) of the canyon are only separated by 250 m but are nevertheless exposed to drastically different microclimates. The remaining two genome assemblies are from Tibetan wild barley. We used high-confidence gene models of barley cv. Morex to find homologous gene-like sequences for all four wild barley genome assemblies. We then scrutinized all identifiable syntenic regions and selected specific loci for in-depth analysis of very recent pseudogenization events in cultivated and wild barley accessions. While assembly quality and sequencing depth differ and complicate genome-wide analysis and statistics, individual gene–pseudogene examples illustrate typical pseudogenization scenarios in closely related subspecies.

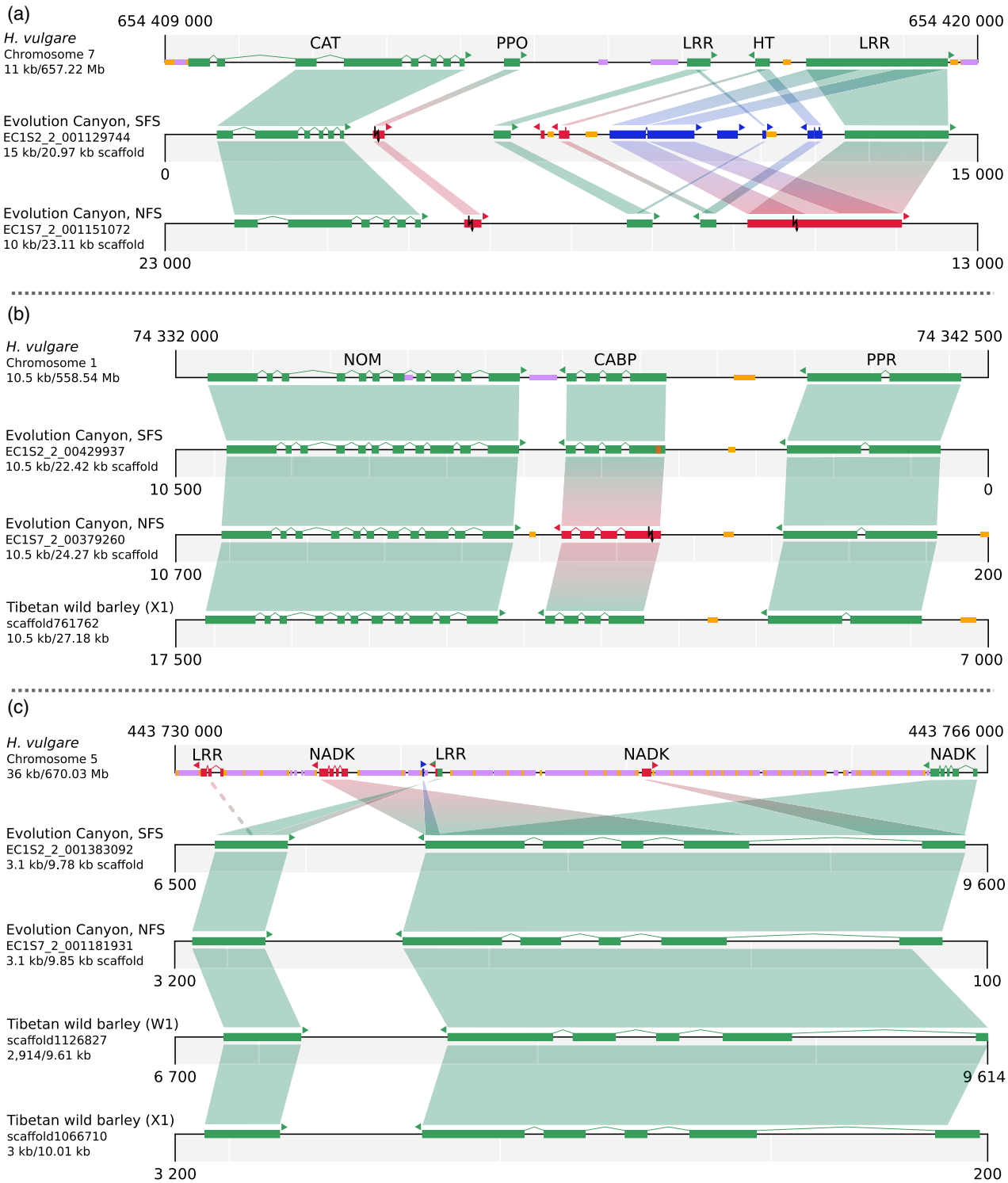
We found a duplicated gene triplet in the wild barley accession growing on the SFS of the Evolution Canyon (Figure 7a). The redundant gene copies contain deletions and insertions, leading to shifts in the reading frame and to premature stop codons. Interestingly, the same triplet is neither duplicated in barley cv. Morex nor the wild barley line growing on the NFS of the Evolution Canyon. The

gene triplet codes for two leucine-rich repeat receptor-like protein kinases (LRR-RK) and a hexosyltransferase (HT). LRR-RKs comprise a large protein family in plants and regulate developmental and defense-related processes (Torii, 2004). The longer of the two *LRR-RK* genes in the wild NFS barley line shows a 13-bp deletion compared with the homologue in barley cv. Morex. This deletion is located in the 5′ half of the coding sequence, resulting in a frameshift and premature stop codons, therefore massively disrupting the derived amino acid sequence. Another gene in the same syntenic region, but not part of the duplicated triplet, is a polyphenol oxidase (*PPO*) gene, which became a pseudogene in both wild barley populations from the Evolution Canyon but is found intact in the cultivated barley sequence. Again, a frameshift leads to premature termination codons. Plant PPOs are enzymes responsible for the browning reaction following tissue damage (Tran *et al.*, 2012). They have been suggested to take part in defense-response mechanisms. Another syntenic region harboring a potential unitary pseudogene contains a calcium-binding protein (*CABP*) gene, which is pseudogenized in one wild barley line (Figure 7b). In humans, CABPs have been shown to be important regulators of key calcium influx channels which are enriched in neuronal tissue (Haynes *et al.*, 2012). In plants, calcium is an important messenger of external signal transduction cascades and as such plays an essential role in the reaction of plants to external stimuli, such as pathogen attack (Poovaiah *et al.*, 1993). In chloroplasts, calcium is involved in photosynthesis, carbon fixation, CO_2 fixation, protein transport and protein phosphorylation (Rocha and Vothknecht, 2013). The homologues of this *CABP* gene in both wild barley lines from the Evolution Canyon exhibit a 1-bp deletion at the beginning of the coding sequence. This results in a frameshift and leads to premature stop codons in the NFS accession. However, another 1-bp insertion in the SFS accession restores the correct reading frame. The most parsimonious sequence of events is that the *CABP* gene in the SFS accession first pseudogenized and was subsequently restored by a counteracting mutation. This example illustrates the continuous transitions between genic and pseudogenic states, which can sometimes even lead to small stretches of drastically changed protein sequence by the transitionally out-of-frame sequence. The described events could well represent a common mechanism for the introduction of novelty and highlight pseudogenes as a 'playground for innovations'.

Transposable elements occupy over 80% of the genomic space in barley and have a strong impact on genome structure. Duplications or rearrangements are often a consequence of mobilization and insertion of transposons. We found a greatly expanded genomic region in barley cv. Morex which experienced insertions of repetitive elements resulting in rearrangements, duplications and pseudogenization (Figure 7c). While in all four wild barley accessions the syntenic LRR and NADH kinase (*NADK*) genes are only separated by about 500 bp, the respective functional copies in cultivated barley span over 20 000 bp, a 40-fold increase in size. To what extent this massive difference is attributable to underlying assembly problems remains speculative for the time being. However, the bordering gene-containing regions exhibit differences to the barley cv. Morex genome and are thus indicative of pseudogenization. The functional *NADK* gene is intact over its entire length, but the LRR gene is shortened due to a frameshift and premature stop codon. However, it still can be regarded as a functioning protein-coding gene. There is another copy of the *NADK* gene which is split into two elements, probably due to repetitive element insertion into the intron of the duplicate. Both fragments are pseudogenized and contain premature termination codons. In conclusion, this region probably experienced massive expansion, rearrangements and duplications leading to pseudogenization in barley cv. Morex, while in all four wild barley genomes this region is largely similar. Even though assembly differences cannot be excluded, it might also indicate that transposable element insertions, pseudogene generation and rearrangements in this region occurred during or after domestication less than 10 000 years ago.

DISCUSSION

The barley genome is rich in full-length HC pseudogenes and numerous small gene fragments. While it comprises 39 734 high-confidence gene loci (Mascher *et al.*, 2017), we found more than twice as many pseudogenes and gene fragments (89 440). A major source of pseudogenes seems to be unequal crossing-over leading to tandem genes. This is affirmed by their retained exon–intron structure, their gene-like chromosomal distribution and the small distance to their respective parent genes. In mammals, retroposed pseudogenes were found to outnumber duplicated pseudogenes (Podlaha and Zhang, 2010; Sisu *et al.*, 2014). In barley, retrotransposition plays only a marginal role in the creation of pseudogenes. This is surprising, because more than 75% of the barley genome is composed of LTR retrotransposons, including about 25 000 full-length and potentially active elements (Spannagl *et al.*, 2013). In comparison, only 45% of the human genome is derived from transposable elements, including 8% LTR-retrotransposons and large amounts (33.7%) of non-LTR-retrotransposons, specifically 16% LINE-1 (L1) retrotransposons (Cordaux and Batzer, 2009). Thus, differences in the prevalence of retroposed pseudogenes cannot be explained by the differential repetitiousness of the genomes but may be linked to the transposable element composition. The enzymatic machinery of LINEs is responsible for the generation of human processed pseudogenes (Pavlicek *et al.*, 2006). In



barley, LINE retrotransposons comprise less than 1% of the genome (Mascher *et al.*, 2017), which could explain the small numbers of retroposed pseudogenes despite the high overall retrotransposon content. The dominance of duplicated pseudogenes over retroposed pseudogenes is not unique for barley; it has also been observed in other plants such as *A. thaliana* and rice (Thibaud-Nissen *et al.*, 2009; Wang *et al.*, 2012).

The non-random chromosomal distribution of retroposed pseudogenes suggests that the reverse transcription

Figure 7. Three syntenic regions containing pseudogenes in barley cv. Morex and four wild barley accessions.
Chromosomal regions are displayed with gene coding sequences (green), pseudogenes with a premature stop codon (red) and potential pseudogenes without a premature stop codon (blue). Syntenic elements are connected. Stretches of undefined sequences (Ns) in the sequence are highlighted in orange; the annotation of repetitive elements on the barley cv. Morex chromosomes is highlighted in violet.
(a) A tandem duplication in one of the Evolution Canyon (EC) accessions. Wild barley from the south-facing slope (SFS) experienced a tandem gene duplication event with a subsequent pseudogenization of redundant copies. A respective leucine-rich repeat (*LRR*) gene in wild barley from the north-facing slope (NFS) contains a 13-bp deletion in the first half of the coding sequence resulting in a frameshift and premature stop codons (lightning symbol). Both wild barley populations share another pseudogene with premature stop codons resulting from a frameshift.
(b) The shifted reading frame of a calcium-binding protein (*CABP*) gene is restored in the SFS accession, but not the NFS accession. A 1-bp deletion is present in both EC accessions, but only the frame of the gene from the SFS accession is restored due to another 1-bp insertion. The shifted region is marked in orange and does not contain premature termination codons.
(c) Transposable elements result in rearrangements and pseudogene creation in barley cv. Morex. The region in barley cv. Morex is greatly expanded (×12 scale difference) due to insertion of repetitive elements resulting in duplications and rearrangements. Copied gene fragments are degenerated. A copy of a *LRR* gene is shortened due to a frameshift. The pseudogene and gene connected with a dashed line have no sequence similarity but were both detected through their homology with different isoforms of the same gene. Gene name abbreviations: catalase (*CAT*), polyphenol oxidase, chloroplastic (*PPO*), hexosyltransferase (*HT*), nucleolar MIF4G domain-containing protein (*NOM*), pentatricopeptide repeat-containing protein (*PPR*), NAD(H) kinase (*NADK*).

of mRNA may not take place exclusively outside the nucleus. The combination of LINE reverse transcription on a chromosomal primer at a nick site (Kaessmann *et al.*, 2009) with the capacity of LINEs to package cellular RNA (Mandal *et al.*, 2013) leads us to propose that LINEs may be responsible for the preferential distribution of retroposed pseudogenes in close spatial proximity to the gene from which they are derived. This LINE-based mechanism would also be consistent with the differential proportions of retroposed pseudogenes in the human and barley genomes. The Rabl conformation of barley chromosomes (Dong and Jiang, 1998; Mascher *et al.*, 2017) results in a neighboring arrangement of short and long chromosome arms in the interphase nucleus. Structural constraints imposed by this configuration could support a preferential reinsertion of retroposed pseudogenes on the opposing chromosome arm to the respective parent gene.

Most of the 89 440 barley pseudogenes are small gene fragments and probably constitute common domains present in high copy numbers. Double-strand DNA break repair mechanisms, so-called non-homologous DNA end joining (NHEJ) or synthesis-dependent strand annealing (SDSA), might be responsible for these short gene fragments, as they are associated with the insertion of filler DNA at the break sites (Gorbunova and Levy, 1997, 1999; Wicker *et al.*, 2010). These processes do not target genes specifically, rendering these short gene fragments symptomatic of the repair mechanism. We found evidence for non-random duplication and pseudogenization preferences, especially for genes in tandem clusters as well as for genes in large or expanded gene families in barley. High duplication rates may be beneficial for rapid adaptation to environmental changes but might also escape dosage compensation mechanisms and thus might be harmful.

We scrutinized syntenic regions between barley cv. Morex and four wild barley accessions for differences in their pseudogene complements and found tandem gene duplications, pseudogenization and sequence rearrangements between the closely related subspecies. However, more detailed comparative analyses were hampered by differences in assembly qualities. While only short contig assemblies were available for the wild barley accessions, the BAC-by-BAC genome assembly of barley cv. Morex provides more complete chromosome sequences. With improved assemblies available in the near future these limitations will be overcome and more detailed comparative analyses between wild and domesticated species and cultivars will become feasible.

CONCLUSION

With the availability of an increasing number of genome reference assemblies, comparative analyses become feasible for plants with large and complex genome structures. The barley genome has recently been sequenced and assembled into chromosomal pseudomolecules, enabling us to perform a whole-genome assessment of pseudogenes. We found almost 90 000 pseudogenes and gene fragments whose analysis sheds light on gene evolution and genome dynamics. There are not only significant differences in pseudogenes between mammals and plants but also between closely related species. The pseudogene complement in domesticated barley and among subspecies growing in different microclimates was found to differ. The main obstacles for comparative analyses remain the qualities of assembly and annotation. Further studies and conclusions about the effect and origin of pseudogenes in the evolution and domestication of crop plants will soon be possible, providing an exciting opportunity.

EXPERIMENTAL PROCEDURES

The identification of pseudogenes was done computationally by exploiting their sequence homology to functional genes. To achieve this, the high-confidence gene set of barley cv. Morex was used as a reference to identify gene-like sequences in the genome. Pseudogenes overlapping with high-confidence genes or with transposable element sequences were filtered.

First, the Morex barley pseudomolecules (Mascher *et al.*, 2017) were split into batches to allow for parallel processing. Transposable elements and transposon genes (Mascher *et al.*, 2017) were N-masked to reduce non-specific hits. The nucleotide CDS of all high-confidence gene isoforms (Mascher *et al.*, 2017) (39 734 loci,

248 180 isoforms) that had no indication of being related to transposable elements (38 157 loci, 240 113 isoforms) were then mapped onto the genome sequence using BLAT (Kent, 2002) (minimal identity 70%, maximum intron length 2500 bp), which creates spliced alignments and thus recovers the exon–intron structures. Short BLAT hits with a length shorter than 50 bp or containing only fragments (exons) shorter than 25 bp were filtered. Gaps (introns) up to a size of 9 bp were closed and considered in the calculation of sequence identity. Premature termination codons were then determined independently for each pseudogene exon, always starting in the correct frame of the parent gene.

Gene self-hits as well as hits overlapping with other high-confidence genes were filtered out completely, but were used to determine whether a gene is a shortened copy of another gene. Non-specific hits, as well as hits with low information content, were filtered using the WU-BLAST dust filtering (Gish, n.d.) (default settings) and the Tandem Repeats Finder (Benson, 1999) (maximum 65% masked, ≥50 bp remaining). If BLAT hits overlapped, the longest hit was chosen as a representative for the locus. If multiple hits with the same maximum length were present at one locus, then the one with the highest sequence identity to its parent was chosen as representative. If the representative covered less than 60% of the locus then all hits shorter than half of the representative and overlapping with it were removed, as well as the hit with the shortest exon length but also the longest total length. This allowed the hit cluster to split up into multiple loci and newly determined representatives to be of good quality. A final filtering step removed BLAT hits from genes with 50 or more children. Those genes were strongly suspected to be related to transposable elements.

The low-confidence gene set of barley contains about 41 000 gene-like sequences that do not fulfill the criteria for canonical genes, including potential pseudogenes. At least 50% of the CDS of 1863 annotated low-confidence genes (4.6%) overlapped with a pseudogene.

Pseudogene classification

The presence or absence of intron sequences in pseudogenes was used to classify them into duplicated or retroposed pseudogenes. Since not all pseudogenes are complete gene copies, some do not span over splice sites, rendering this type of classification impossible. For the intron loss/retention criterion, we defined five pseudogene classes (Figure S1): (i) 'duplicated' pseudogenes still containing introns at each covered splice site; (ii) 'retroposed' or 'processed' pseudogenes which have lost all introns; (iii) 'chimeric' pseudogenes with both retained and lost introns; (iv) 'single-exon parent' pseudogenes from isoforms with only one exon; (v) 'fragmented' pseudogenes which do not sufficiently cover a splice site. A splice site is only covered if at least 10 bp of the exons on either side are present in the duplicate. The gap has to be at least 30-bp long, to be considered as a duplicated intron.

Chromosomal distribution of pseudogenes and other elements

Densities of genes, pseudogenes and transposons along the chromosomes where calculated with a sliding window of 5 megabases (Mb) and a shift size of 1 Mb as percentage sequence coverage. Circular figures were created using Circos v.0.69-4 (Krzywinski *et al.*, 2009).

K_a/K_s analysis

To determine the selection pressure on pseudogenes, the sequences of pseudogene/parent gene pairs need to be aligned and edited. We used clustalw2 (Larkin *et al.*, 2007) (default) for pairwise alignment and removed codons containing gaps or undefined nucleotides (Ns), as well as premature termination codons. The alignment was always kept in the frame of the gene. In order for the subsequent analysis to work correctly, a minimum alignment length of 150 bp was a pre-condition. CodemI from the PAML package (Yang, 2007) was used to calculate K_a and K_s values. Highly similar sequences led to extreme $\log_{10}$ K_a/K_s values (e.g. ≥99). For the statistical analysis, we filtered for log-values between −4 and +4 (32 021 $\log_{10}$ K_a/K_s values remained after all filtering steps). We used the scipy 'normaltest' from python to test for a normal distribution and the scipy one-sample *t*-test 'ttest_1samp' to test whether the distribution is significantly shifted from the expected mean of zero.

Gene families and orthologous groups

Gene families were determined by first using BLAST (Altschul *et al.*, 1990) (blastn) on the representative gene splice variants with an E-value threshold of 1×10^{-5}. Then mcxdeblast was used and its output forwarded to mcl (van Dongen, 2000; Enright *et al.*, 2002). Orthologous groups were defined from the barley high-confidence class genes and the annotated gene sets of three grasses from diverse grass subfamilies (*Sorghum bicolor*, *Brachypodium distachyon* and *Oryza sativa*) and *A. thaliana* using OrthoMCL software v.2.0 (OrthoMCL default parameters). A total of 170 925 CDS from these five species were clustered into 24 337 gene families. Of these clusters, 8608 contained sequences from all five genomes. Expanded gene families were extracted as described in Mascher *et al.* (2017).

Gene Ontology analysis

To find under- or over-represented Gene Ontology (GO) terms in the parent gene set compared with the complete gene set (sub-ontology: biological process), we used the free open-source GOstats R package (Falcon and Gentleman, 2007) with a *P*-value cutoff of 0.05. The resulting GO terms were then grouped with REVIGO (Supek *et al.*, 2011) using a similarity threshold of 0.5 and *A. thaliana* as the GO term database.

RNA-seq analysis

Hisat2 was used to align RNA-seq reads (Mascher *et al.*, 2017) to the barley genome (options: –dta-cufflinks). Samfiles were then filtered for a minimal mapping quality value of 60, converted into BAM files and sorted using Samtools (v.1.3). Cufflinks and Cuffcompare (2.2.1) were then used to assemble alignment files to a single set of transcripts. It was then checked whether there was transcriptional evidence for pseudogenes and for HC pseudogenes in particular. A pseudogene was considered to be transcribed if at least 50 bp of its sequence overlapped with transcription evidence in either direction.

Shortened genes within the gene set

BLAT hits, which were filtered because they overlapped with annotated genes, were used to determine whether a gene is a shortened copy of another gene. A pre-condition was that the homology of the shortened gene to the longer gene extended beyond its own CDS. The shorter gene had to be at least 60% covered by the hit, with either less than 60% of the hit overlapping with the short gene or the hit being at least 100 bp longer than the short gene at that position.

Tandem genes and pseudogenes

Coding sequences of genes were clustered using CD-HIT (Li and Godzik, 2006; Fu *et al.*, 2012) (80% identity threshold) and tandem gene groups were then defined from the resulting clusters by applying a maximum distance requirement of 50 000 bp between any pair of genes. Pseudogene children of tandem genes were considered part of the tandem group if their distance to any of its gene members did not exceed 50 000 bp as well.

Syntenic regions between cultivated and wild barley lines

We used four wild barley genome assemblies (Tan *et al.*, 2017) to investigate differences in gene and pseudogene complements between the two closely related species. Filtering for contigs and scaffolds with a minimum length of 200 bp and a maximum of 35% Ns was performed in an attempt to remove bad-quality sequences. We then used an equivalent of the pseudogene detection pipeline to map the representative isoform of our domesticated barley gene CDS (Mascher *et al.*, 2017) onto the four assemblies. The resulting hits formed a collection of genes and pseudogenes which all have a parent gene homologue from the Morex barley gene set. Hits were classified as genes if they met all following requirements: (i) nucleotide differences must not lead to premature termination codons shortening the CDS by more than 15 nucleotides; (ii) their sequence identity compared with the Morex homologue is at least 95%; (iii) the CDS of the Morex homologue is covered to at least 98% if the hit has a length smaller than 800 bp, otherwise it has to be covered to at least 75%. This very stringent definition led to low gene numbers, which is why the remaining hits were divided into pseudogenes with premature stop codons and potential pseudogenes without premature stop codons. Often, potential pseudogenes were located at the borders of a scaffold, resulting in shortened annotations and low coverage. To be able to better estimate whether an element is a gene or a pseudogene, elements of interest were individually examined and aligned to their parent gene using megablast or blastn (Altschul *et al.*, 1990). To investigate syntenic blocks, we focused on and visualized contigs and scaffolds which contain at least three genes with homologues on a maximum stretch of 1 Mb of the same Morex *H. vulgare* chromosome. Since the sequence data for the wild barley populations from the Evolution Canyon were a combination of two assembly versions, possible duplicates of the same locus were removed in the visualizations. The sequence of the higher-quality assembly version was kept. A pairwise comparative visualization of syntenic blocks was created between Morex barley and each of the four wild barleys, if available. A CD-HIT clustering (95% identity, 80% coverage in both directions) of the CDS of the Morex query gene was used to determine the connections of syntenic genes or pseudogenes. Any element pair from the same cluster is connected in the visualization. This resulted in over 800 syntenic block pairs. If they share at least one gene with Morex barley, syntenic block figures were then combined to allow for the comparison of more than two barley lines. The resulting 203 shared syntenic blocks were manually scrutinized and three loci of interest were selected.

Availability of data and material

The data generated and analyzed during the current study are available from the PGSB ftp site at ftp://plantftp.helmholtz-muenchen.de/barley/. Sequence data for wild barley accessions have been deposited in the sequence read archive (SRA) with the SRA identifier SRP076351.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

VMP analyzed and interpreted the data. HG provided the transposon annotation and interpreted the data. ST performed the alignment and assembly of the RNA-seq reads. ST provided the gene annotation. NS, RW, CL, GZ, PL, AHS, RW and MP provided pre-publication access to the assembly and transcription data. CL, BC and CT contributed to the sequences of the wild barley accessions. VMP, KFXM and HG wrote the paper. All authors read, contributed to and approved the final manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Computational pseudogene detection and classification pipeline.

Figure S2. Sequence identity versus distance of pseudogenes compared with their parent genes.

Figure S3. Distribution of transposable elements, genes and intrachromosomally retroposed pseudogenes on the seven chromosomes of barley.

Figure S4. Over- and under-represented Gene Ontology terms for the parent gene set of transcribed pseudogenes compared with the complete gene set of the barley genome.

Figure S5. Analysis of the relative rates of synonymous and non-synonymous substitutions between pseudogenes and their parent genes.

Table S1. Metrics for pseudogenes and high-coverage pseudogenes, as well as template and parent genes in the barley genome.

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