Development of a New A<sup>m</sup>–Genome-Specific Single Nucleotide Polymorphism Marker Set for the Molecular Characterization of Wheat–*Triticum monococcum* Introgression Lines

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Cultivated einkorn wheat (*Triticum monococcum* L. subsp. *monococcum*, 2<sup>n</sup> = 2<sup>x</sup> = 14, A<sup>A</sup>M<sup>a</sup>) and its wild relative *T. monococcum* subsp. *aegilopoides* are important sources of economically useful genes that can be exploited for wheat (*Triticum aestivum* L.) breeding. Einkorn has excellent resistance to fungal diseases and gene transfer is relatively simple via standard breeding methods. To fulfill the growing demand by modern prebreeding programs for a cost-effective high-throughput procedure for accurately detecting introgressed chromosomes or chromosome segments from *T. monococcum* into wheat, we used the Axiom Wheat-Relative Genotyping Array and developed a set of A<sup>A</sup>m genome-specific exome-based single nucleotide polymorphism (SNP) markers suitable for rapid identification of *T. monococcum* chromatin in a wheat background. We identified 1247 polymorphic SNPs between *T. monococcum* and wheat. We identified 191 markers validated across all seven chromosomes of *T. monococcum* that are also present on an existing *Triticum urartu* Thum. ex Gandil. genetic map and potentially ordered them on the basis of the high macrocollinearity and conservation of marker order between *T. monococcum* and *T. urartu*. The marker set has been tested on leaf-rust-resistant BC<sub>3</sub>F<sub>4</sub> progenies of wheat–*T. monococcum* hybrids. Two markers (AX-94492165, AX-95073542) placed on the distal end of the chromosome arm 7AL detected a *T. monococcum* introgression into wheat. The SNP marker set thus proved highly effective in the identification of *T. monococcum* chromatin in a wheat background, offering a reliable method for screening and selecting wheat–*T. monococcum* introgression lines, a procedure that could significantly speed up prebreeding programs.

**Abbreviations:** FISH, fluorescent in situ hybridization; SNP, single nucleotide polymorphism.


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14, A\textsuperscript{m}A\textsuperscript{m}), commonly known as einkorn, was domestici-
dated from its wild form (\textit{T. monococcum} subsp. \textit{agilopi-
doides}) about 12,000 yr ago (Heun et al., 1997). \textit{Triticum}
\textit{monococcum} is closely related to \textit{T. urartu} (A\textsuperscript{a}A\textsuperscript{a}; John-
sen and Dhaliwal, 1976), which is the A genome progeni-
tor of durum and bread wheat (Dvořák \textit{et al.}, 1993), with the A\textsuperscript{a} and A\textsuperscript{m} genomes diverging 0.5 to 1 million years
ago (Huang \textit{et al.}, 2002). It has been reported that the
A\textsuperscript{m} and A\textsuperscript{a} genomes have a high level of gene collinear-
ity (Devos \textit{et al.}, 1995); however, molecular differences
have also been found (Wicker \textit{et al.}, 2003). Chromosomal
distribution of the sequence identity and comparative
analysis of genes between the A subgenome of wheat and
those of A genome lineages (\textit{T. urartu} and \textit{T. monococ-
cum}) showed that high sequence similarity and struc-
tural conservation are retained, with limited gene loss
and chromosomal rearrangement (International Wheat
Genome Sequencing Consortium, 2014; Marcussen \textit{et al.},
2014). High sequence similarity between \textit{T. monococcum}
and \textit{T. urartu} also allowed Fox \textit{et al.} (2014) to map 95% of
the 120,911 exome transcripts of \textit{T. monococcum} to the \textit{T. urartu}
geno{me} (Ling \textit{et al.}, 2013) successfully.

Today, einkorn is cultivated only marginally to pro-
duce traditional or organic products but it harbors many
important genes that can be used in wheat breeding
(Munns \textit{et al.}, 2012). \textit{Triticum monococcum}, belonging to
the primary gene pool of wheat, has excellent resistance
to diseases such as leaf rust (\textit{Puccinia triticina}), stem rust,
yellow rust, and powdery mildew and several resistance
genes [\textit{Lr} 10, (leaf rust resistance); \textit{Sr}21, \textit{Sr}22, and \textit{Sr}35
(stem rust resistance); and \textit{Pm}25 and \textit{Pm}26 (powdery
mildew resistance)] have been mapped and transferred
to bread wheat (Zaharieva and Monneveux, 2014). Gene
transfer from the primary gene pool is relatively simple
and is based on standard breeding methods such as
homologous recombination, hybridization, and back-
crossing (Mujeet-Kazi and Rajaram, 2002).

The identification of introgressed chromatin in
wheat–ancestral hybrids and backcrossed progenies is a
crucial step in the prebreeding process. Fluorescent in
situ hybridization (FISH) with repetitive DNA probes
made it possible to discriminate between the chro-
nomes of the \textit{T. monococcum} A\textsuperscript{m} genome and the
chromosomes of the A genome of wheat (Badaeva \textit{et al.},
2015; Megyeri \textit{et al.}, 2017). Nevertheless, this method is
limited for identifying small segments of \textit{T. monococcum}
chromatin in a wheat background. Therefore, the develop-
ment of high-throughput molecular markers covering
the entire A\textsuperscript{m} genome is essential for uncovering and
detecting new wheat–\textit{T. monococcum} introgression lines.

Genetic maps of einkorn involving restriction frag-
ment length polymorphism markers, isozymes, seed
storage proteins, rRNA, and morphological loci have
been reported before (Dubcovsky \textit{et al.}, 1996; Taenzler
\textit{et al.}, 2002). Simple sequence repeat markers developed in
hexaploid wheat have also been mapped on to \textit{T. mon-
ococcum} chromosomes, resulting in the construction of an
integrated molecular linkage map of the A\textsuperscript{m} genome of \textit{T.}
\textit{monococcum} (Fricano \textit{et al.}, 2014; Hammer \textit{et al.}, 2000;
Singh \textit{et al.}, 2007). Genetic linkage maps using Diversity
Arrays Technology markers have also been reported for
\textit{T. monococcum} (Jing \textit{et al.}, 2009; Marino \textit{et al.}, 2018).

Some of these linkage mapping studies in \textit{T. monococ-
cum} compared their genetic maps to the physical map of
the \textit{T. urartu} genome and reported that there was a high
degree of marker order conservation between A\textsuperscript{a} and A\textsuperscript{m}
chromosomes (Fricano \textit{et al.}, 2014; Marino \textit{et al.}, 2018).
However, these markers are low-throughput or have
limited success in the wheat background and thus are
not suitable for the characterization and identification
of wheat–\textit{T. monococcum} recombinant chromosomes in
large-scale pre-breeding programs.

Here, we present a set of exome-based SNP markers
specific to the \textit{T. monococcum} genome that have proven
to be effective in the precise identification of new wheat–\textit{T.}
\textit{monococcum} introgression lines. We used the Axiom Wheat-
Relative Genotyping Array (Affymetrix, Santa Clara, CA)
and the allele calling procedure described by King \textit{et al.}
(2017) to identify 1247 polymorphic SNPs between wheat and
\textit{T. monococcum}. From among these markers, we selected 191
high-quality SNP markers that have been validated on wheat–
\textit{T. urartu} backcrossed progenies. These could be used as diag-
nostic markers for detection of \textit{T. monococcum} introgressions in
a wheat background.

\section*{MATERIALS AND METHODS}

\subsection*{Plant Materials}

One leaf-rust-resistant accession of diploid \textit{T. monococ-
cum} subsp. \textit{monococcum} (MVGB1306, obtained from
Gene Bank of Martonvasar) was used to produce a
wheat–\textit{T. monococcum} interspecific F\textsubscript{1} hybrid. The hybrid
was backcrossed with the wheat parent (cultivar Mv9kr1)
to generate BC\textsubscript{1}, BC\textsubscript{2}, and BC\textsubscript{3} populations (Molnár-
Láng \textit{et al.}, 1996). The BC\textsubscript{3} plants were self-fertilized
in the greenhouse to produce the BC\textsubscript{3}F\textsubscript{2} generation used for the leaf rust
resistance tests. Resistant plants were self-fertilized
three times and the BC\textsubscript{3}F\textsubscript{2} generation was genotyped by
the Axiom Wheat-Relative Genotyping SNP array. A \textit{T. urartu}
genetic map developed previously (Grewal \textit{et al.},
2018) was used in the present study to select and validate
\textit{T. monococcum} chromosome-specific SNP markers.

\subsection*{Evaluation of Leaf Rust Resistance}

Artificial leaf rust inoculation was performed in a green-
house with a uredospore suspension on 65 \textit{T. aestivum} \times
\textit{T. monococcum} BC\textsubscript{3}F\textsubscript{1} plants during the 2012–2013
growth season. The plants were inoculated at the two-leaf
stage and infection types were recorded on the 10th day
after inoculation as described by Stakman \textit{et al.} (1962).

\subsection*{Genotyping via an Axiom SNP Array and Selection
of \textit{T. monococcum} Genome-Specific Markers}

DNA samples were genotyped by the Axiom Wheat-
Relative Genotyping Array as described by King \textit{et al.}
(2017). The procedure is documented by Affymetrix.
(https://assets.thermofisher.com/TFS-Assets/LSG/manuals/axiom_genotyping_solution_analysis_guide.pdf, accessed 12 June 2019). Eight BC$_3$F$_4$ wheat–T. monococcum plants originating from the two leaf-rust-resistant BC$_3$F$_1$ plants (four progeny randomly selected from each parent) and the parental lines (wheat cultivar Mv9kr1 and T. monococcum) were genotyped together with the wheat cultivar Paragon, the T. urartu parent and 258 samples of the wheat–T. urartu backcrossed populations developed by Grewal et al. (2018).

After genotyping with the array, the SNPs were classified into categories as follows: (i) ‘Poly High Resolution’, with at least two examples of the minor allele; (ii) ‘No Minor Homozygote’, with two clusters observed; (iii) ‘Off-Target Variant’, which had four clusters, one representing a null allele; (iv) ‘Mono High Resolution’, which were monomorphic; (v) ‘Call Rate Below Threshold’, where the SNP call rate was below the threshold but other cluster properties were above the threshold; and (vi) ‘Other’, where one or more cluster properties were below the threshold (Hussain et al., 2017). To select the chromosome-specific SNPs, the highest quality Poly High Resolution SNPs were used, as they provided three well-resolved genotype clusters. Flapjack (Milne et al., 2010) was used to remove any SNP markers where (i) either or both parents were clustered as heterozygous calls, (ii) both the wheat and the wild relative parents (T. monococcum, T. urartu) were clustered together in the same genotype (i.e., no polymorphism) and/or (iii) the parental lines had an undetermined genotype. The polymorphic markers were assigned to T. monococcum chromosomes according to information from the genetic map of T. urartu (Grewal et al., 2018).

Comparative Analysis

T. monococcum genome-specific markers, also present on the T. urartu map, were used in BLASTN (E-value: 10$^{-5}$) analysis against the wheat genome reference assembly RefSeq version 1 (International Wheat Genome Sequencing Consortium et al., 2018) and the T. urartu genome reference assembly (Ling et al., 2018). The results were visualized (Fig. 1) with MapChart version 2.32 (Voorrips, 2002).

RESULTS

Response of T. aestivum × T. monococcum BC$_3$F$_1$ Seedlings to Leaf Rust Disease

Sixty-five BC$_3$F$_1$ wheat–T. monococcum seedlings were randomly selected and their response to leaf rust infection was recorded on the 10th day after inoculation (Table 1). Two of the 65 seedlings showed the same immunity as the T. monococcum parent, whereas most of the remaining plants (n = 55) were very susceptible, similar to the wheat parental line.

Selection and Validation of T. monococcum Chromosome-Specific SNP Markers

In total, 18,287 SNPs that were polymorphic between T. urartu and wheat and distributed across all seven chromosome groups in wheat were included on the Axiom Wheat-Relative Genotyping array (Winfield et al., 2016). In the present work, we screened DNA from T. monococcum, T. urartu, Mv9kr1 and Paragon wheat, eight BC$_3$F$_4$ lines originating from the wheat-T. monococcum leaf-rust-resistant hybrid, and 258 lines obtained from the wheat–T. urartu backcross population (Grewal et al., 2018). The Axiom ‘SNPolisher’ R package allocated the scores for each of the markers into six cluster patterns (Hussain et al., 2017); however, only the calls classified as Poly High Resolution SNPs (3168 SNPs) represented good quality cluster resolution and were thus included in the genotyping. Of these, 1247 high-quality SNPs were selected as polymorphic between wheat and T. monococcum by Flapjack and were physically mapped to the A genome of wheat via a BLASTN search (Supplemental Table S1; International Wheat Genome Sequencing Consortium et al., 2018). From these 1247 T. monococcum SNP markers, 191 were identified as being in common with the T. urartu genetic map (Grewal et al., 2018) and were thus selected as a set of validated high-quality T. monococcum chromosome-specific SNP markers (Table 2). The lowest numbers of SNPs with wheat were detected on homeologous Group 1 (8.9%); homeologous Group 5 (23%) showed the highest number of SNPs.

The physical position of the 191 marker sequences was found on the wheat A genome (International Wheat Genome Sequencing Consortium et al., 2018) and on the T. urartu genome (Ling et al., 2018) via a BLASTN search (Supplemental Table S2). On the basis of previous work that suggested high synteny and collinearity between T. monococcum and T. urartu (Fricano et al., 2014; Marino et al., 2018), the 191 SNP markers were tentatively ordered on the T. monococcum chromosomes according to their physical position on the T. urartu genome (Fig. 1). Triticum monococcum-specific markers also showed macro synteny with the A genome of wheat (Fig. 1), except in the case of chromosome 4A, which has a pericentric inversion in polyploid wheat that is not found in its diploid progenitor, T. urartu (Devos et al., 1995).

From the BC$_3$F$_2$ progenies of the two leaf-rust-resistant plants, eight individuals were randomly selected (four from each parent) and screened with the Axiom Wheat-Relative Genotyping array, which resulted in the detection of a single T. monococcum introgression. Two T. monococcum-specific markers (AX-94492165 and AX-95073542), located within 200 bp of each other on the telomeric region of chromosome arm 7AL (Fig. 1), were detected in each of the analyzed plants. The sequences of these two markers were used in a BLASTX search against the wheat genome (International Wheat Genome Sequencing Consortium et al., 2018; http://plants.ensembl.org/Triticum_aestivum/Info/Index, accessed 7 June 2019) to determine any potential candidate genes. However, the immediate flanking regions of the markers did not show any annotated genes.
Fig. 1. Comparison of physical positions of T. monococcum chromosome-specific single nucleotide polymorphism markers on T. urartu chromosomes (Ling et al., 2018) and the A genome of wheat (IWGSC et al., 2018) across the seven homeologous groups. All positions are indicated as 1 × 10^7 bp.
DISCUSSION

The genetic diversity of einkorn wheat provides a promising opportunity to improve the resistance of bread wheat against a wide spectrum of fungal pathogens (Zaharieva and Monneveux, 2014). To speed up the gene transfer from an alien species into wheat, it is essential to precisely trace the transferred chromosome segments in the progenies. The development of array-based chromosome-specific marker sets represent a cost-effective, high-throughput solution to accurately identify the introgressed chromosome segments within modern pre-breeding programs.

The A genome of wheat and those of its diploid relatives (the $A^a$ and $A^m$ genomes) share a remarkably similar chromosomal gene content, whereby gene sequences are highly conserved, reaching 98% identity (International Wheat Genome Sequencing Consortium, 2014; Marcussen et al., 2014). A previous study by Marino et al. (2018) compared the genetic map of T. monococcum and the shotgun assemblies of the T. urartu genome (Fox et al., 2014) and the bread wheat genome (Clavijo et al., 2017) and reported a high degree of conservation of marker order between them, since most markers found in the same position or in close proximity in T. monococcum were aligned to the same contig in T. urartu and bread wheat. The main exception was chromosome 4A, which carries the well-known pericentric inversion (Devos et al., 1995; Dvorak et al., 2018; Mickelson-Young et al., 1995), which is consistent with the BLAST results from this study (Fig. 1). In the comparison of T. monococcum and the bread wheat genome assembly (Marino et al., 2018), only a few markers for each linkage group (average: 7.2%) were mapped in different bread wheat chromosomes. Grewal et al. (2018) genetically mapped 368 exome-based SNP markers into seven linkage groups in T. urartu and compared them, via BLAST, to the bread wheat genome, reporting that only six markers (1.6%) were located on different A genome chromosomes in wheat.

In the present study, we developed a polymorphic SNP marker set between T. monococcum and wheat. By using the recently published wheat reference assembly (International Wheat Genome Sequencing Consortium et al., 2018) and the T. urartu genome assembly (Ling et al., 2018) and exploiting the possible macrocollinearity (Fricano et al., 2014; Marino et al., 2018) among T. urartu ($A^aA^a$), T. monococcum ($A^mA^m$), and the A genome of wheat, we were able to potentially hypothesize the order of the markers within the T. monococcum chromosomes (Fig. 1). However, a more accurate approach to identify the order of gene-specific SNP markers along chromosomes is single-gene FISH, which can be applied on mitotic metaphase chromosomes. Single-gene FISH, together with FISH using repetitive sequences, is useful in chromosome identification and allows investigation of chromosome rearrangements and comparative studies on chromosome structure between species with the A genome lineage (Danilova et al., 2014; Said et al., 2018).

Anker and Niks (2001) identified a large number of einkorn accessions that were resistant to leaf rust. Leaf-rust-resistant accessions originated in a higher proportion from T. monococcum subsp. monococcum than from T. urartu and T. monococcum subsp. aegilopoides. Genes from leaf rust resistance have been transferred into wheat from T. monococcum subsp. monococcum and are located on chromosomes 2A, 3A, and 5A (Dyck and Bartoš, 1994; Kaur et al., 2008; Singh et al., 2007). In this study, we identified two leaf-rust-resistant individuals in the progenies of a wheat–T. monococcum F$_1$ hybrid and indicated that the resistance could potentially be associated with the presence of two T. monococcum-specific SNP markers located on the telomeric region of chromosome

<table>
<thead>
<tr>
<th>Homeologous Group</th>
<th>Number of SNP markers</th>
<th>Percentage of total SNP markers</th>
<th>Validated SNPs on the T. urartu genetic map</th>
<th>Percentage of total SNPs on the T. urartu genetic map</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homeologous Group 1</td>
<td>118</td>
<td>9.5</td>
<td>17</td>
<td>8.9</td>
</tr>
<tr>
<td>Homeologous Group 2</td>
<td>208</td>
<td>16.7</td>
<td>34</td>
<td>17.8</td>
</tr>
<tr>
<td>Homeologous Group 3</td>
<td>211</td>
<td>16.9</td>
<td>21</td>
<td>11.0</td>
</tr>
<tr>
<td>Homeologous Group 4</td>
<td>155</td>
<td>12.4</td>
<td>27</td>
<td>14.1</td>
</tr>
<tr>
<td>Homeologous Group 5</td>
<td>234</td>
<td>18.8</td>
<td>44</td>
<td>23.0</td>
</tr>
<tr>
<td>Homeologous Group 6</td>
<td>121</td>
<td>9.7</td>
<td>22</td>
<td>11.5</td>
</tr>
<tr>
<td>Homeologous Group 7</td>
<td>200</td>
<td>16.0</td>
<td>26</td>
<td>13.6</td>
</tr>
<tr>
<td>Total</td>
<td>1247</td>
<td>100</td>
<td>191</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1. Response to leaf rust in 65 T. aestivum × T. monococcum BC$_3$F$_1$ plants at the seedling stage.

<table>
<thead>
<tr>
<th>Infection types</th>
<th>No. of inoculated T. aestivum × T. monococcum BC$_3$F$_1$ plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very susceptible</td>
<td>55</td>
</tr>
<tr>
<td>Moderately susceptible</td>
<td>5</td>
</tr>
<tr>
<td>Moderately resistant</td>
<td>1</td>
</tr>
<tr>
<td>Very resistant</td>
<td>1</td>
</tr>
<tr>
<td>Nearly immune</td>
<td>1</td>
</tr>
<tr>
<td>Immune</td>
<td>2</td>
</tr>
</tbody>
</table>
arm 7A of L. The Lr20–Sr15–Pm1 resistance locus identified historically in hexaploid wheat that confers resistance to three different fungal wheat pathogens has also been mapped to the distal part of wheat chromosome 7AL (Neu et al., 2002; Sears and Briggie, 1969). Jayatilake et al. (2013) developed expressed sequence tag markers (wr1, wr2, wr3, wr4, and wr5) between the Lr20/ Sr15 locus and the phytoene synthase gene (Psy-A1) and all markers colocated with the Lr20 gene. These markers are placed on the distal region of chromosome arm 7AL from 724,135,301 bp (wr1) to 726,482,191 bp (wr5) (International Wheat Genome Sequencing Consortium Refseq version 1.0). The T. monococcum genome-specific markers presented in this study are positioned between 700,275,508 bp (AX-94492165) to 700,275,682 bp (AX-95073542) on chromosome arm 7AL (International Wheat Genome Sequencing Consortium Refseq version 1.0). Neu et al. (2002) and Jayatilake et al. (2013) have suggested that Lr20 is in a region where recombination is suppressed. They proposed that this could be caused by an alien introgression or a genetic rearrangement. Our results indicate that a recombination event has taken place on the telomeric region of 7AL chromosome arm, resulting in the introgression of a short T. monococcum chromosome segment into the wheat background, carrying an effective leaf rust resistance gene acting in the seedling stage.

The wheat–T. monococcum SNP marker set developed and validated in the present study offers accurate, cost-effective, and high-throughput detection of T. monococcum chromatin in a wheat background and thus significantly speeds up the transfer of valuable traits from a wild relative into bread wheat in modern breeding programs.

Supplemental Information

Supplemental Table S1. The sequence information of 1247 polymorphic SNPs between T. monococcum and wheat.

Supplemental Table S2. BLASTN results detailing the physical positions of the T. monococcum specific marker set on the A genome of wheat (RefSeq version 1; International Wheat Genome Sequencing Consortium et al., 2018) and the T. urartu pseudomolecules (Ling et al., 2018).

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by the Marie Skłodowska-Curie Fellowship Grant (H2020-MSCA-IF-2016-752453-LANDRACES) from the European Union; the Biotechnology and Biological Sciences Research Council [grant number BB/P016855/1], as part of the Designing Future Wheat Programme; and the Hungarian National Research, Development and Innovation Office (K129221). The funding body played no role in the design of the study and collection, analysis, and interpretation of data or in writing the manuscript. We also thank Dr. Amanda Burridge and Mr. Paul Wilkinson from University of Bristol for their invaluable help in genotyping and bioinformatics.

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