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Authors

Gabay, Gilad
Zhang, Junli
Burguener, German Federico
et al.

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ORIGINAL RESEARCH

Structural rearrangements in wheat (1BS)–rye (1RS) recombinant chromosomes affect gene dosage and root length

Gilad Gabay[1] | **Junli Zhang**[1] | **German Federico Burguener**[1] | **Tyson Howell**[1] | **Hanchao Wang**[1,2] | **Tzion Fahima**[2] | **Adam Lukaszewski**[3] | **Jorge Ignacio Moriconi**[4] | **Guillermo Esteban Santa Maria**[4] | **Jorge Dubcovsky**[1,5]

[1] Department of Plant Sciences, University of California, Davis, CA 95616, USA

[2] Department of Evolutionary and Environmental Biology and the Institute of Evolution, University of Haifa, Haifa, Israel

[3] Department of Botany & Plant Sciences, University of California, Riverside, CA 92521, USA

[4] Instituto Tecnológico Chascomús (INTECH)Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Univ. Nacional de San Martín, Chascomús, Buenos Aires 7130, Argentina

[5] Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA

Correspondence
Jorge Dubcovsky, Department of Plant Sciences, University of California, Davis, CA, 95616, USA.
Email jdubcovsky@ucdavis.edu

[^] The first two authors contributed equally to this work

Abstract

Good understanding of the genes controlling root development is required to engineer root systems better adapted to different soil types. In wheat (*Triticum aestivum* L.), the 1RS.1BL wheat–rye (*Secale cereale* L.) translocation has been associated with improved drought tolerance and a large root system. However, an isogenic line carrying an interstitial segment from wheat chromosome arm 1BS in the distal region of the 1RS arm ($1RS^{RW}$) showed reduced grain yield and shorter roots both in the field and in hydroponic cultures relative to isogenic lines with the complete 1RS arm. In this study, we used exome capture to characterize $1RS^{RW}$ and its parental lines T-9 and 1B+40. We show that $1RS^{RW}$ has a 7.0 Mb duplicated 1RS region and a 4.8 Mb 1BS insertion colinear with the 1RS duplication, resulting in triplicated genes. Lines homozygous for $1RS^{RW}$ have short seminal roots, while lines heterozygous for this chromosome have roots of intermediate length. By contrast, near-isogenic lines carrying only the 1BS distal region or the 1RS-1BS duplication have long seminal roots similar to 1RS, suggesting a limited effect of the 1BS genes. These results suggest that the dosage of duplicated 1RS genes is critical for seminal root length. An induced deletion encompassing 38 orthologous wheat and rye duplicated genes restored root length and confirmed the importance of gene dosage in the short-root phenotype. We explored the expression profiles and functional annotation of these genes and discuss their potential as candidate genes for the regulation of seminal root length in wheat.

1 | INTRODUCTION

The translocation of the short arm of rye (*Secale cereale* L.) chromosome 1 (1RS) from the cultivar Petkus into the long arm of wheat (*Triticum aestivum* L.) chromosome 1B (henceforth, 1RS.1BL) confers improved tolerance to several abiotic and biotic stresses. Although several genes for resistance to biotic stresses are no longer effective, the

Abbreviations: $1RS^{RW}$, distal segment of wheat 1BS chromosome inserted in the 1RS arm; $1RS^{WW}$, distal and proximal segments of wheat 1BS chromosome inserted in the 1RS arm; CIMMYT, Centro International de Mejoramiento de Maíz y Trigo; CS, Chinese Spring; EMS, ethyl methanesulfonate; kb, kilobase; N-Del, nonhomozygous for the deletion; RAM, root apical meristem; RNASeq, RNA-sequencing; SNP, single nucleotide polymorphism.

1RS.1BL translocation is still widely used because of its beneficial effects on grain yield (Kim, Johnson, Baenziger, Lukaszewski, & Gaines, 2004; Shearman, Sylvester-Bradley, Scott, & Foulkes, 2005) and improved abiotic stress tolerance (Carver & Rayburn, 1994; Ehdaie, Layne, & Waines, 2012; Hoffmann, 2008; Moreno-Sevilla, Baenziger, Peterson, Graybosch, & Mcvey, 1995; Schlegel & Korzun, 1997; Villareal, Rajaram, Mujeebkazi, & Deltoro, 1991; Zarco-Hernandez, Santiveri, Michelena, & Pena, 2005).

We have previously shown that the presence of a short segment of wheat 1BS chromosome from cultivar Pavon in the distal region of the 1RS translocation (henceforth $1RS^{RW}$) was associated with reduced grain yield, biomass, and canopy water status relative to near-isogenic lines carrying the complete 1RS chromosome arm (Howell et al., 2014 , 2019). Carbon isotope discrimination data showed that the lines with the complete 1RS chromosome arm achieve higher yields and better water status through increased access to water throughout the season, rather than through water conservation (Howell et al., 2014).

A subsequent field study showed that the improved water status of the isogenic lines with the 1RS chromosome was associated with enhanced root density below 20 cm relative to the lines with the $1RS^{RW}$ chromosome (Howell et al., 2019). Changes in root architecture in the field were correlated with drastic changes in root development in hydroponic growth systems, where the $1RS^{RW}$ line showed a regulated arrest of the seminal root apical meristem (RAM) ~2 wk after germination. By the same time, the $1RS^{RW}$ plants displayed altered gradients of reactive oxygen species in the root tips and emergence of lateral roots close to the RAM (Howell et al., 2019).

In this study, we performed exome captures for 1RS, $1RS^{RW}$, and its parental lines T-9 (distal 1BS segment) and 1B+40 (distal 1RS segment). We show that, as a result of a distal inversion between 1RS and 1BS chromosome arms, T-9 and 1B+40 have duplicated 1BS and 1RS orthologous regions in opposite orientations and that a crossover between these chromosomes resulted in a duplicated 1RS region colinear to the inserted 1BS segment in $1RS^{RW}$. Using these genetic stocks, we demonstrate that the dosage of the genes in the duplicated region plays an important role in the regulation of the seminal root growth. We also describe a radiation mutant with a deletion in the inserted 1BS segment and the adjacent 1RS region that restored long roots, confirming the importance of the dosage of the genes in this region on root development. Finally, we identified 38 genes within this deletion and used published RNA-sequencing (RNASeq) data and annotation to discuss their potential as candidates for the genes regulating seminal root elongation in wheat.

Core Ideas

- Distal recombinant chromosomes between wheat 1BS and rye 1RS carry large, duplicated regions.
- An inversion between the distal regions of chromosomes 1BS and 1RS caused the duplications.
- Exome capture data defined the borders of duplicated and recombined regions.
- Changes in gene dosage were associated with changes in root development.
- A radiation deletion defined a region with 38 candidate genes for root length.

2 | MATERIAL AND METHODS

2.1 | Plant materials

The genetic stocks including the 1RS and $1RS^{RW}$ chromosome arms were initially generated in the cultivar Pavon 76 (henceforth Pavon), a spring wheat developed at the International Maize and Wheat Improvement Center (Centro Internacional de Mejoramiento de Maíz y Trigo, CIMMYT) (Lukaszewski, 2000). The 1RS chromosome arm translocation in Pavon was introgressed from the CIMMYT cultivar Genaro, which, in turn, received the translocation from the cultivar Kavkaz (Rajaram, Mann, Qrtiz-Ferrara, & Mujeeb-Kazi, 1983). The donor of the 1RS arm in Kavkaz was the rye cultivar Petkus, one of the leading rye cultivars in the 20th century.

2.1.1 | 'Hahn' lines with different wheat segments inserted into 1RS chromosome arms

To name the different chromosome constitutions we used two superscripts, with the first superscript indicating the proximal position and the second superscript the distal position. The 'R' superscript indicates rye chromatin and the 'W' superscript the wheat chromatin. The $1RS^{RW}$ chromosome arm (synonymous with $1RS_{40:9}$.1BL) was generated by homologous crossover in overlapping wheat segments of the primary 1BS–1RS recombinant T-9, which possessed a distal wheat 1BS segment, and 1B+40, which possessed a distal 1RS segment (Figure 1) (Lukaszewski, 2000). The $1RS^{WR}$ arm (synonymous with $1RS_{44:38}$.1BL) was generated by a crossover in overlapping wheat segments in primary 1BS–1RS recombinants T-38, which possessed a large distal wheat 1BS segment, and 1B+44, which possessed a long distal 1RS segment (Figure 1) (Lukaszewski, 2000). The $1RS^{WW}$ chromosome

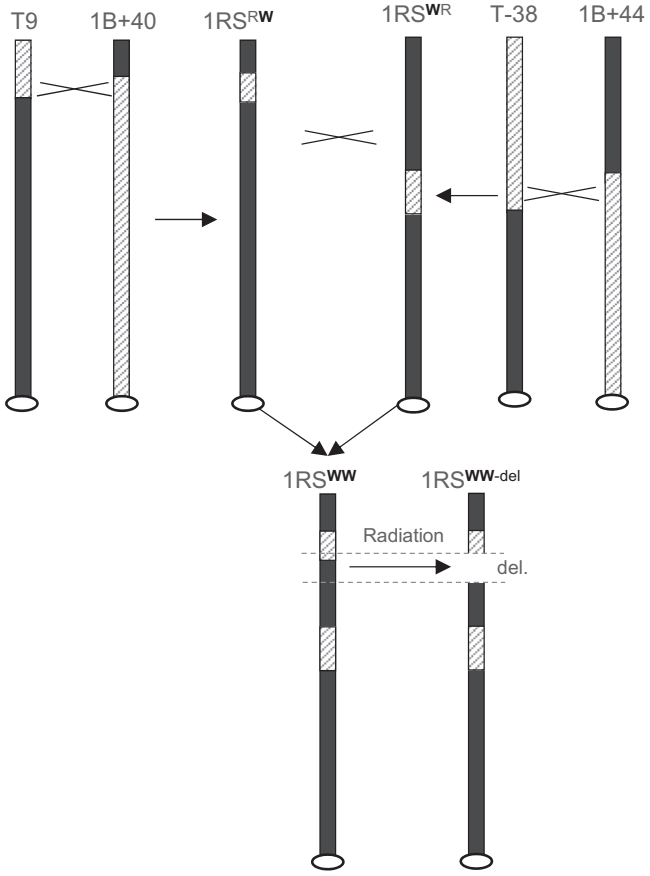

FIGURE 1 Development of the recombinant 1RS/1BS lines used in this study. White rectangles with diagonal lines indicate wheat chromatin and grey rectangles rye chromatin. All lines have a 1BL long arm. The first 'W' in the superscript indicates the proximal wheat segment and the second 'W' the distal wheat segment. $1RS^{WW}$ arm has both segments and was generated by crossing $1RS^{WR}$ and $1RS^{RW}$. These stocks were developed before Lukaszewski (2000), with the exception of the radiation mutant $1RS^{WW\text{-}del}$ developed in this study. For this deletion, two sister lines, designated as $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$, were used

was generated by a crossover between $1RS^{RW}$ and $1RS^{WR}$ chromosomes and was designated as chromosome MA1 in Lukaszewski (2000) (Figure 1).

The lines carrying the $1RS^{RW}$, $1RS^{WR}$, and $1RS^{WW}$ chromosomes were previously backcrossed into the CIMMYT common wheat cultivar Hahn, which has the 1RS.1BL translocation, with 1RS also originating from cultivar Kavkaz, the same as in Pavon-1RS. The introgressions involved six marker-assisted backcrosses, resulting in near-isogenic lines (Howell et al., 2014) that were deposited in the National Small Grains Collection as accessions PI 672839 ($1RS^{RW}$), PI 672838 ($1RS^{WR}$), and PI 672837 ($1RS^{WW}$).

We have previously shown that the $1RS^{RW}$ chromosome results in short roots in the Hahn background but not in the Pavon background. Therefore, to analyze the effects of different 1RS/1BS recombinant chromosomes on root length, we backcrossed primary recombinants with varying lengths of wheat and rye segments—T-9, T-18, T-21, and 1B+40 (Lukaszewski, 2000)—four times into Hahn. Line T-21 is identical to T-9 and line T-18 carries a large distal 1BS segment on its 1RS arm (similar to T-38 in Figure 1) and was used as 1BS reference in the calculation of ratios for copy number determination. Line 1B+37, which carries a large distal 1RS segment on its 1BS arm (similar to 1B+44 in Figure 1), was used as 1RS reference in the exome capture comparisons but was not used in the hydroponic experiments.

2.1.2 | Radiation mutants

To dissect the chromosome region affecting root length, we irradiated 5,000 wheat F_2 seeds from the cross between Hahn × Hahn-$1RS^{WW}$ with 300 Gy (from a Cesium-137 source at the Center for Health and the Environment at University of California–Davis). This mutant population was established in $1RS^{WW}$ (Figure 1) before we knew which wheat segment was affecting root length. The objective of mutagenizing F_2 plants rather than homozygous plants was to detect deletion mutants in the heterozygous plants of the first generation without having to wait for progeny tests.

We extracted DNA from the 2,200 mutagenized plants that survived and used a dominant wheat marker (wPt1911) and a dominant rye molecular marker (o-sec-up/low) (Supplemental Table S1) to eliminate plants that were homozygous for the 1RS or 1BS segments. We identified 907 plants that were heterozygous for the proximal segment, of which, we expected the majority to also be heterozygous for the distal 1BS segment. We then screened the selected plants with multiple markers for the distal 1BS insertion and identified one mutant (Figure 1). From the progeny of this plant, we selected two sister homozygous plants, designated hereafter as $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$. We then backcrossed these two deletions independently to Hahn-$1RS^{RW}$ and to Hahn (1RS) four times to reduce background mutations and to test the effect of the deletion on the root length in both backgrounds. Although the two lines carry the same deletion, independent backcrosses increase chances of eliminating different background mutations, and they served as biological replicates in the root length experiments.

2.2 | Exome capture and copy number variation

We performed two exome capture experiments using different platforms. In the wheat exome capture using the assay developed by Arbor Biosciences, we included lines T-9, T-18, T-21, 1B+37, 1B+40, and $1RS^{RW}$ ethyl methanesulfonate (EMS) mutant lines RW_M4_43_11 and RW_M4_47_12 (we used their average as $1RS^{RW}$ in the different copy

number analyses). In the wheat exome capture using the assay developed by NimbleGen (Krasileva et al., 2017), we included lines 1RS, $1RS^{RW}$, and deletion lines $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$. Based on the average similarity between the wheat and rye genes (>90%) and the hybridization conditions used in the capture, we expect most of the rye genes to be captured with both wheat exome capture assays.

The exome captures were sequenced using the Illumina platform and 150 bp paired-end reads at the University of California, Genome Center. The sequencing reads were preprocessed to trim adapters with Trimmomatic v0.39 (Bolger, Lohse, & Usadel, 2014). Since the capture included both wheat and rye reads, we mapped the reads to a combined reference including wheat Chinese Spring (CS) RefSeq v1.0 chromosome 1B and the rye chromosome arm $1RS^{AK58}$ from the 1RS.1BL translocation in cultivar Aikang58 (Ru et al., 2020). To minimize off-target mapping, we mapped the reads at high-stringency with 'bwa aln' v0.7.16a-r1181 (Li & Durbin, 2009), allowing only perfectly mapped reads (zero single nucleotide polymorphisms [SNPs]). Alignments were sorted by using samtools v1.7 (Li et al., 2009), and duplicate reads were removed with Picard tools v2.7.1 (http://broadinstitute.github.io/picard/).

We normalized the number of mapped reads so that all lines have the same total number of reads mapped to the chromosome arm 1BL. We selected the 1BL arm as reference because 1RS/1BS recombinant lines differ in their short arm constitutions, but all share identical 1BL arms. We then calculated normalized read number ratios using a common reference line (1RS or 1B+37 for 1RS and T-18 for 1BS). We generated heat maps for these ratios and visually determined the borders of duplication, recombination, and deletion events. We then validated these borders using *t* tests of the ratios at both sides of the border (we used average ratios per gene as replications). For these analyses we excluded genes with less than six reads in the accessions used as denominator for normalization.

We report wheat gene coordinates using CS RefSeq v1.1 (International Wheat Genome Sequencing Consortium et al., 2018) and rye gene coordinates using the $1RS^{AK58}$ genome as references (Ru et al., 2020), which is almost identical to our 1RS sequence. The other available genome reference for rye inbred line Lo7 (Rabanus-Wallace et al., 2019) is less similar to the 1RS sequences from Hahn 1RS.1BL translocation.

2.3 | Phenotyping

Hydroponic experiments were performed in growth chambers at 22–23 ˚C with a photoperiod of 16 h light vs. 8 h dark (fluorescent lights supplemented with incandescent lighting). In all experiments, grains were imbibed at 4 ˚C for 4 d and then placed at room temperature. Once the coleoptiles emerged, seedlings were floated on a mesh to develop roots for 4 d. After removing the grain, seedlings were wrapped at the crown with foam and inserted in holes precut in a foam core board placed on top of the solution. The detail protocols and solutions are described in our previous paper (Howell et al., 2019).

As in our previous study, experiments in this study were performed in two different laboratories in Argentina and the United States using tanks of 0.35 and 13 L, respectively. As a result of the different conditions, final root lengths differed across experiments. However, differences among genotypes were consistent across experiments, and all statistical comparisons among genotypes were performed within experiment or using experiments as blocks. In experiments performed in 13-L tanks, we changed nutrient solution every 3 d and we included all genotypes in each tank. When necessary, we used multiple tanks as blocks. In experiments performed in 0.35-L tanks, we changed nutrient solution every 2 d, and a single genotype was included per pot, with multiple pots used as replications.

To determine the effect of the $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ deletions on root development, we evaluated the segregating plants in the BC_2F_2 and BC_4F_2 generations to account for potential random effects of residual deletions in other chromosomes.

3 | RESULTS

In this study we characterized a set of recombinant 1BS–1RS chromosomes carrying different deleted and duplicated chromosome segments and tested their phenotypic effect on root architecture in hydroponic experiments. Since the interpretation of these root phenotypes requires a clear understanding of the genetic stocks used in these experiments, we describe first the genetic rearrangements present in these lines and then their effect on the root phenotypes.

3.1 | The 1BS chromosome segment in $1RS^{RW}$ is 4.8 Mb long and includes 115 genes

To define the borders of the inserted 1BS region, we used the Arbor Biosciences exome capture to characterize the $1RS^{RW}$ line and its two parental lines 1B+40 (distal 1RS) and T-9 (distal 1BS; Supplemental Table S2). We also included line T-21 that appears to be identical to T-9 (as a replicate), line T-18 that has a distal 1BS segment longer than T-9/T-21 and was used as a wheat reference, and line 1B+37 that has a longer distal 1RS segment than 1B+40 and was used as a rye reference. We mapped the reads of each capture to a combined reference (CS RefSeq v1.1 chromosome 1B and $1RS^{AK58}$) without allowing any SNP and then normalized the counts to a similar number of mapped reads per capture in the 1BL arm.

For the analysis of the 1BS region, we divided the number of normalized reads in each line by the normalized number of reads of T-18, which was used as the 1BS reference (up to 17 Mb). This analysis showed that the distal 1BS border was the same in 1B+40 and $1RS^{RW}$ and was located between coordinates 4,791,410 and 4,811,515 bp in the CS 1BS pseudomolecule (henceforth 4.8 Mb). Statistical tests confirmed highly significant differences in the ratios at both sides of the breakpoint ($P < .001$) for $1RS^{RW}$ and 1B+40 and no significant differences for T-9 and T-21 (Supplemental Figure S1a). The proximal 1BS border was the same in T-9, T-21, and $1RS^{RW}$ and was located between 9,551,729–9,554,904 bp (henceforth 9.6 Mb). Statistical tests confirmed highly significant differences in the ratios at both sides of 9.6 Mb ($P < .001$) for T-9, T-21, and $1RS^{RW}$ but not for 1B+40 (Supplemental Figure S1b). Based on these results, we estimated that the 1BS segment inserted in $1RS^{RW}$ is 4.8 Mb long and includes 115 annotated high-confidence genes from *TraesCS1B02G009700* to *TraesCS1B02G020300* (CS RefSeq v1.1 annotation, Supplemental Figures S1a and S1b).

3.2 | The 1BS chromosome segment did not replace the orthologous 1RS genes in $1RS^{RW}$

The recent sequencing of the 1RS arm (Ru et al., 2020) revealed the presence of a large inversion between the distal region of chromosome arms 1RS (telomere to 13.875 Mb) and 1BS (telomere to 15.579 Mb; Figure 2a), which suggests that lines with breakpoints within this region, such as T-9, T-21 and 1B+40, may be more complex than originally thought. The $1RS^{RW}$ line was generated by a crossover of the primary recombinant lines T-9 (distal 1BS segment in a 1RS arm) and 1B+40 (distal 1RS segment in a 1BS arm) (Lukaszewski, 2000), and the previous results indicate that $1RS^{RW}$ has retained the 1RS-1BS breakpoints of T-9 and 1B+40 (Figure 2a). The $1RS^{RW}$ chromosome arm also has the same strong telomeric C-band as 1RS and 1B+40, indicating that it has retained the complete 1RS segment present in 1B+40 (Figure 2a, distal black rectangle).

We initially assumed that the 1BS segment in $1RS^{RW}$ replaced the orthologous rye genes and that the loss of these genes could be responsible for the shorter roots of Hahn-$1RS^{RW}$. However, the codominant marker THdw11 has both the 1RS ($1RS^{AK58}$: 6.57 Mb) and 1BS bands (CS: 8.171 Mb) in T-9, 1B+40, and $1RS^{RW}$ but not in T-18 or 1B+37 (Figure 2b), suggesting a duplication rather than a replacement in the lines with distal crossover events. To investigate the extent of this duplication, we first identified 14 orthologous 1BS-1RS gene pairs including high-confidence wheat genes located within the 1BS insertion and rye $1RS^{AK58}$ genes that were at least 90% identical with an aligned region covering >90% of the gene (Table 1). Surprisingly, all 14 rye-orthologues were present in the exome capture of T-9, 1B+40, and $1RS^{RW}$ (Table 1), which indicated that the complete rye region orthologous to the 1BS insertion was present in these lines. Since no 1RS gene was missing in the 1BS orthologous region, we rejected the hypothesis that lost rye genes were responsible for the differences in root length between Hahn-1RS and Hahn-$1RS^{RW}$ isogenic lines.

The presence of this large duplicated 1BS-1RS colinear region in both T-9 (distal 1BS) and 1B+40 (distal 1RS) was particularly surprising given their different chromosomal configurations. To understand the structure of these chromosomes, we first determined the borders of the 1RS region in T-9 and 1B+40 by mapping the exome capture reads at high stringency (zero SNPs) to the combined CS RefSeq v1.1 chromosome 1B and $1RS^{AK58}$. The first 1RS border proximal to the 1BS insertion in T-9 and T-21 was located approximately between 3,096,772 and 3,151,733 bp (henceforth, 3.1 Mb) in the $1RS^{AK58}$ genome and it was conserved in $1RS^{RW}$ (Figure 2a; Supplemental Figure S2). The second 1RS border distal to the 1BS insertion in 1B+40 was located approximately between 10,071,332 and 10,079,335 bp (henceforth, 10.1 Mb) in the $1RS^{AK58}$ genome (Figure 2a; Supplemental Figure S2). Analyses of the 1RS and 1BS border regions suggest that the breakpoints in both T-9 (= T-21) and 1B+40 involved orthologous regions in the 1BS and 1RS genomes (Supplemental Figure S3), which is expected since these lines were generated using the *ph1b* mutation that promotes homoeologous recombination (Lukaszewski, 2000).

A more detailed analysis of the 1RS/1BS breakpoint region in 1B+40 revealed the presence of a 0.7–1.2 Mb secondary inversion in the border of the 1RS segment nested within the large 13.875 Mb inversion. Within this secondary inversion the centromere to telomere orientation is the same in 1RS and 1BS. This explains why the crossover in this region did not generate a dicentric chromosome and acentric fragment, which is expected from a crossover event within an inverted region (Supplemental Figure S3). Except for this small (0.7–1.2 Mb) secondary inversion, the rest of the genes in the distal 1RS segment are in the opposite orientation to the order of the genes in the 1BS segment, explaining the duplication of the genes in this region.

The analysis of the breakpoint region in T-9 failed to reveal any obvious secondary inversion (Supplemental Figure S3), but we cannot rule out the possibility of small inversions affecting a few genes since we do not have the complete genome of wheat cultivar Pavon, which is the source of the 1BS segment. We hypothesize that, similarly to what we observed in 1B+40, a small secondary inversion within the large 13.875 Mb inversion may have generated a small region with a common centromere–telomere orientation facilitating the origin of the T-9 and T-21 recombinant chromosomes. As in 1B+40, we predict that most genes in the 1BS and 1RS segments at both sides of the breakpoint point in T-9 and T-21 are

TABLE 1 Wheat genes in the 1BS inserted segment and their orthologues in $1RS^{AK58}$ present in $1RS^{RW}$ using a stringent threshold (>90% identity and >90% coverage). Gene names in bold, TraesCS1B02G010400 to TraesCS1B02G020000, are present within the 1BS segment in $1RS^{RW}$. One or two genes outside the inserted 1BS segment are included as reference. Underlined coordinates indicate genes within the borders of the regions deleted in $1RS^{WW\text{-}del\text{-}8/10}$

	Wheat 1BS RefSeq v1.1			BLAST Rye $1RS^{AK58}$					1BS–1RS		
Gene name	**Chromosome**	**Start**	**End**	**Chromosome**	**Start**	**End**	**Identity**	**Coverage**	**T-9**	**1B+40**	**$1RS^{RW}$**
TraesCS1B02G							——%——				
008000	1BS	4,345,220	4,352,718	1RS	9,944,445	9,938,961	91	98.8	B+/R+	**B−**/R+	**B−**/R+
010400	**1BS**	4,965,664	4,966,572	1RS	8,809,005	8,809,910	90	100	B+/R+	B+/R+	B+/R+
011200	**1BS**	5,159,471	5,159,817	1RS	8,529,282	8,529,618	90	97	B+/R+	B+/R+	B+/R+
011400	**1BS**	5,177,355	5,177,573	1RS	8,520,902	8,520,684	94	100	B+/R+	B+/R+	B+/R+
011500	**1BS**	5,509,787	5,510,370	1RS	8,490,379	8,489,797	91	100	B+/R+	B+/R+	B+/R+
015300	**1BS**	7,337,194	7,338,438	1RS	7,033,744	7,032,500	91	100	B+/R+	B+/R+	B+/R+
016400	**1BS**	7,988,894	7,995,986	1RS	8,464,852	8,457,806	95	94	B+/R+	B+/R+	B+/R+
017200	**1BS**	8,249,060	8,255,234	1RS	6,438,623	6,432,318	92	100	B+/R+	B+/R+	B+/R+
017300	**1BS**	8,255,692	8,257,056	1RS	6,431,734	6,430,497	90	91	B+/R+	B+/R+	B+/R+
017800	**1BS**	8,663,173	8,677,747	1RS	5,249,402	5,253,169	96[1]	99.7	B+/R+	B+/R+	B+/R+
018500	**1BS**	8,853,375	8,854,210	1RS	4,549,151	4,549,986	94	100	B+/R+	B+/R+	B+/R+
018600[a]	**1BS**	8,864,601	8,867,261	1RS	4,515,291	4,513,815	94	100	B+/R+	B+/R+	B+/R+
019500[a]	**1BS**	9,161,801	9,164,311	1RS	4,483,541	4,482,068	93	100	B+/R+	B+/R+	B+/R+
019900	**1BS**	9,281,178	9,282,051	1RS	4,214,948	4,214,202	93	99.6	B+/R+	B+/R+	B+/R+
020000	**1BS**	9,414,580	9,415,508	1RS	3,997,202	3,998,063	93	99.6	B+/R+	B+/R+	B+/R+
020500	1BS	9,578,944	9,581,744	1RS	3,058,092	3,058,905	94	100	B+/**R−**	B+/R+	**B−**/R+
020700	1BS	9,592,944	9,596,685	1RS	2,488,927	2,486,243	92	98.9	B+/**R−**	B+/R+	**B−**/R+

[a]Excluding exon 2 from 1BS annotated as intron in 1RS (we analyzed the other 12 exons).

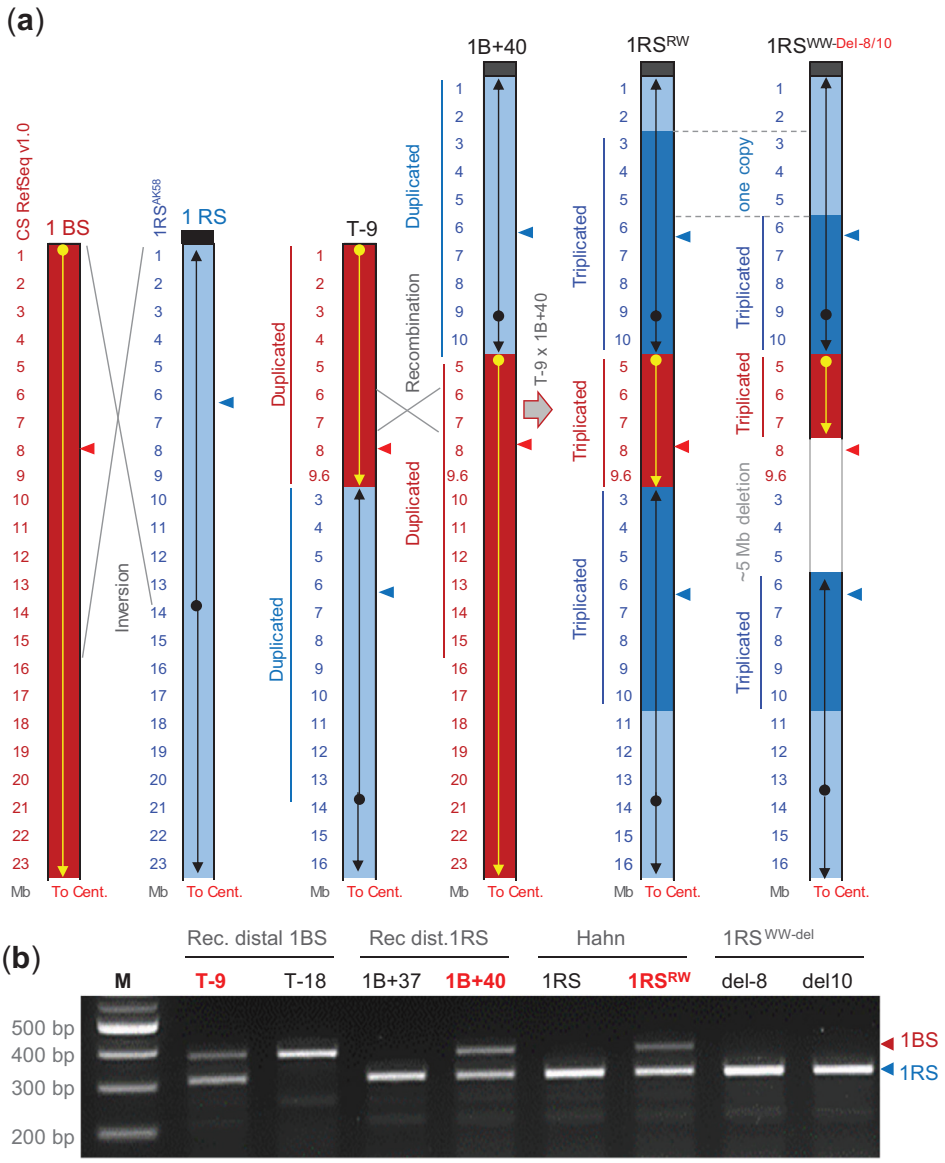

FIGURE 2 Chromosome rearrangements in 1RS/1BS recombinant lines. (a) From left to right: 13.875 Mb inversion between the wheat 1BS (Chinese Spring RefSeq v1.1) and 1RS (AK58) chromosomes. Structural changes in primary-recombinant lines T-9 and 1B+40. $1RS^{RW}$ chromosome formed from the crossover between T-9 × 1B+40. The dotted lines in the radiation mutants generated in $1RS^{WW}$ indicate the maximum deleted regions in the distal 1BS segment (1.56 Mb) and the adjacent 1RS region (3.3 Mb), which include orthologous genes. The proximal wheat insertion in $1RS^{WW\text{-}del\text{-}8/10}$ is located between 17.6 and 26.8 Mb, outside of this figure (see Figure 1). Red numbers are coordinates in the Chinese Spring RefSeq v1.1 and blue numbers in the rye $1RS^{AK58}$ genome (both in Mb). Red is 1BS, light blue is 1RS, and dark blue is the duplicated 1RS region. Arrows within the chromosomes indicate the order of the genes relative to the telomere–centromere order in 1BS. Red and blue arrowheads indicate the position of the THdw11 marker in 1BS and 1RS, respectively. (b) Codominant marker THdw11 (1BS: 8.17 Mb, $1RS^{AK58}$: 6.57 Mb) showing the 1RS-1BS duplication in T-9, 1B+40, and $1RS^{RW}$ and the deletion of the 1BS band (red arrowhead) but not the 1RS band (blue arrowhead) in the deletion lines $1RS^{WW\text{-}del\text{-}8/10}$. Recombinant lines T-18 (17 Mb distal 1BS) and 1B+37 (15 Mb distal 1RS) with crossovers outside the inverted regions do not have the duplication

duplicated and in an inverted order (Figure 2a; Supplemental Figure S3).

Regardless of the mechanism that generated the T-9 and 1B+40 chromosomes, the crossover within the 4.8 Mb of the overlapping 1BS region that originated the $1RS^{RW}$ chromosome (Figures 1 and 2) is expected to generate a duplication of the 1RS segment between 3.1 and 10.1 Mb. This duplication is clearly visible in Supplemental Figure S4 (violet line), where we plotted the ratios between the reads per kilobase (kb) per gene for $1RS^{RW}$/1RS (violet line) from the NimbleGen exome capture experiment vs. the position in $1RS^{AK58}$. Since the 1BS segment inserted in $1RS^{RW}$ is

orthologous to the duplicated 1RS region, all genes in the 1BS segment are triplicated (Figure 2).

As expected, the borders of the $1RS^{RW}$ duplicated region coincide with the borders of the 1BS–1RS breakpoints in T-9 and 1B+40 (Supplemental Figures S2a and S2b). The $1RS^{RW}$/1RS ratios between the normalized number of reads from the exome capture were significantly higher in the region inside the duplication (>3.1 and <10.1 Mb) than in the region outside the duplication (<3.1 and >10.1 Mb, $P < .001$; Supplemental Figures S2a and S2b, respectively). These results support the chromosome models presented in Figure 2a.

3.3 | A radiation mutant showed a 5-Mb deletion of the T-9 border in $1RS^{WW}$

To test if these changes in gene dosage affect root length, we generated a radiation mutant population for $1RS^{WW}$. Among the mutagenized $1RS^{WW}$ plants, we identified only one line that carried a deletion in the critical region. To control for possible background mutations in the phenotypic experiments, we generated two sister lines, designated $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$, using independent backcrosses. For the exome capture, these two lines served as replicates in the determination of the deletion borders (Supplemental Table S3). To study the border of the deletion within the 1BS segment, we compared the ratios $1RS^{RW}$/1BS with the $1RS^{WW\text{-}del\text{-}8/10}$/1BS ratios. We observed a significant drop in the $1RS^{WW\text{-}del\text{-}8/10}$/1BS ratios between the normalized numbers of reads per kb between 7,995,986 and 8,116,677 bp that was not observed in $1RS^{RW}$ (Supplemental Figure S5a). This deletion border was supported by significantly higher ratios in the 1BS region between 4.8 and 7.9 Mb than in the region between 8.2 and the end of the 1BS segment at 9.6 Mb ($P < .001$) in the deletion lines relative to the $1RS^{RW}$ control with a complete 1BS segment (Supplemental Figure S5a).

To determine if the radiation deletion extended into the 1RS segment of $1RS^{RW}$ and to determine its size, we analyzed the ratios of reads/kb in $1RS^{RW}$/1RS, $1RS^{RW\text{-}del\text{-}8}$/1RS, and $1RS^{RW\text{-}del\text{-}10}$/1RS along the $1RS^{AK58}$. These analyses showed that the deletion (3.1–6.0 Mb) was within the 1RS duplicated region (3.1 and 10.1 Mb; Supplemental Figures S4) and that the border of the 1RS deletion was between 6,010,958 and 6,422,733 bp in $1RS^{AK58}$ (Supplemental Figure S5b). This border was supported by significantly smaller ratios in the deleted 1RS region (3.1–6.0 Mb, ratios ~1) than between 6.4 and 10 Mb (ratios ~2, $P < .001$) in the deletion lines but not in the $1RS^{RW}$ control (Supplemental Figures S4 and S5b). Interestingly, since the genes in 1RS and 1BS regions are in inverted orientation around the breakpoint, all genes deleted in the 1RS region have orthologues in the deleted 1BS region (*TraesCS1B02G017200–TraesCS1B02G020000*; Table 1). Wheat genes *TraesCS1B02G017200* and *TraesCS1B02G017300* were deleted in $1RS^{WW\text{-}del\text{-}8/10}$ but their rye orthologues were outside the deleted region (Table 1). Because of the $1RS^{WW\text{-}del\text{-}8/10}$ deletion, the 1RS region between 3.1 and 6.0 Mb is present in one copy in $1RS^{WW\text{-}del\text{-}8/10}$ and in two copies in $1RS^{RW}$, which also carries an additional 1BS orthologous copy (Figure 2a).

3.4 | Changes in dosage of the duplicated chromosome regions affect root length

To test the effect of the dosage of the triplicated distal segment on root length, we evaluated 71 F_2 plants from the cross between Hahn-1RS × Hahn-$1RS^{WW}$ described before. The $1RS^{WW}$ chromosome has the same distal duplicated 1RS region (3.1–10.1 Mb) and orthologous 1BS distal segment (4.8–9.6 Mb; Figure 2a) as $1RS^{RW}$ plus a linked proximal wheat insertion that does not affect the root length (Howell et al., 2019). To better represent the number of duplicated rye (R) and wheat (B) genes present in the different lines, we used the formula 4R+2B for the triplicated distal region in $1RS^{RW}$ homozygotes, 2R for 1RS homozygotes, 3R+1B for 1RS/$1RS^{RW}$ heterozygotes, 2B for T-18 homozygotes, and 2B+2R for T-9, T-21, and 1B+40 homozygotes.

Among the 71 F_2 plants included in the hydroponic experiment, we identified 25 homozygotes for the distal 1RS segment, 10 homozygotes for the distal wheat segment, and 36 heterozygotes using the dominant wheat molecular marker THdw04 and the dominant rye molecular marker o-sec-up/low (Supplemental Table S1). This segregation represents a marginally significant deviation for the expected 1:2:1 segregation (χ^2 $P = .042$). The roots of plants homozygous for $1RS^{WW}$ (4R+2B) were 21.3 cm shorter ($P < .001$; Figure 3a) than the roots of the plants homozygous for the distal 1RS segment (2R), confirming the results reported in our previous study (Howell et al., 2019). The heterozygotes (3R+1B) showed an intermediate root length that was significantly different from both homozygotes in a Tukey test. The average root length of heterozygous plants was 2.8 cm longer than the midpoint indicated by a violet dotted line (44.3 cm, 26% dominance; Figure 3a). This result suggests that the dosage of the genes within the triplicated region in the $1RS^{RW}$ plants has an effect on root length.

To test the effect of different dosages of the distal region, we first compared root lengths of Hahn plants homozygous for the 1RS, $1RS^{RW}$, 1B+40, and T-21 chromosomes (BC_4F_2 to BC_5F_2). We used the T-21 introgression into Hahn and not the T-9 because the backcrossing of T-21 was more advanced and both lines have indistinguishable 1RS–1BS breakpoints (Supplemental Figures S2 and S3). In this experiment, the roots of the T-21 and 1B+40 lines were slightly shorter than those in 1RS (combined average 2.4 cm shorter than 1RS, with T-21 slightly shorter than 1B+40) but the difference was not

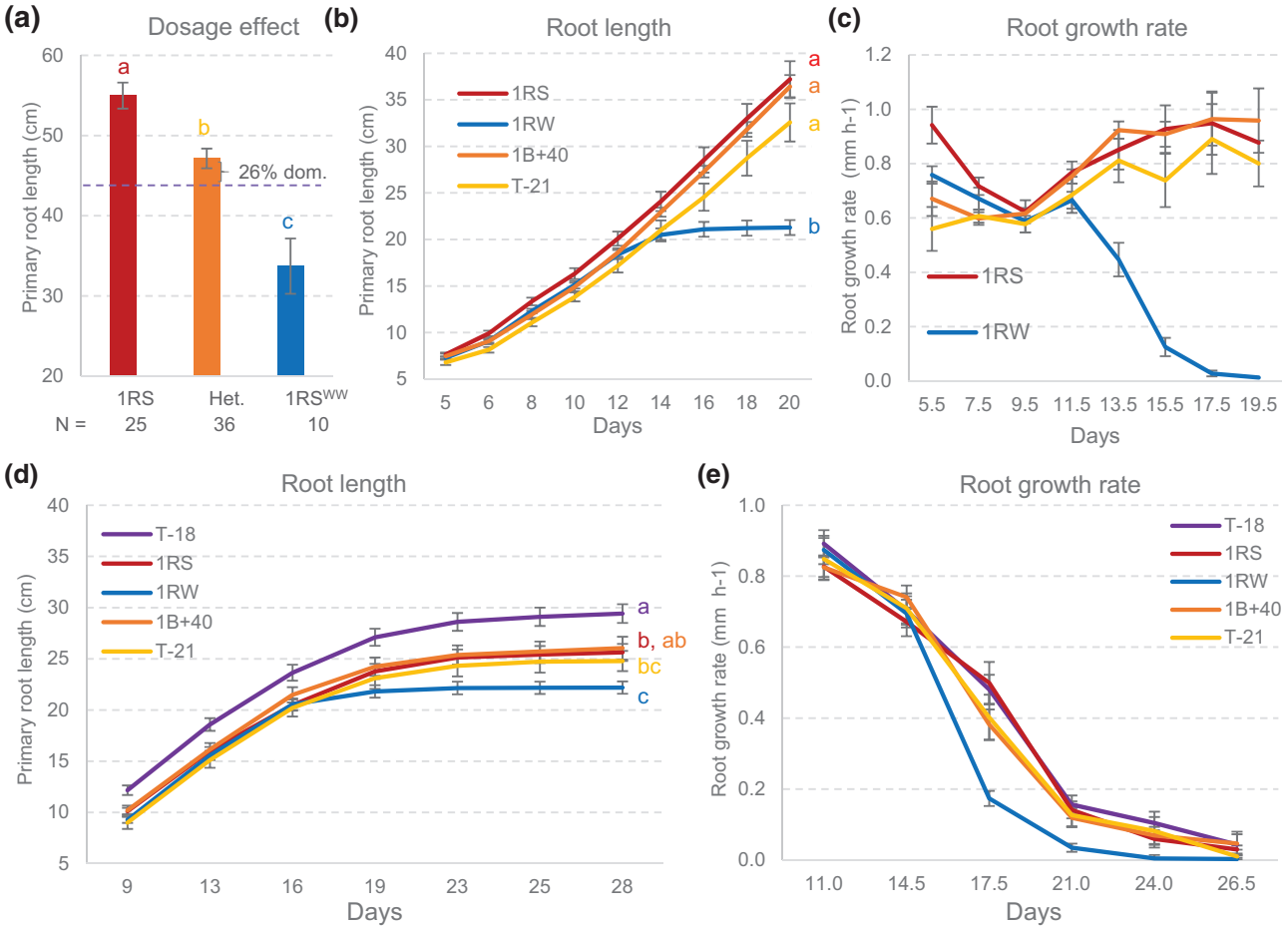

FIGURE 3 Effect of gene dosage on root length. (a) Root length in the F_2 population Hahn-1RS × Hahn-1RS^{WW}, numbers in parentheses in the title indicate the dosage of the duplicated region in different genomes (R = rye 1RS and B = wheat 1BS). We calculated the degree of dominance based on Falconer (1964). (b) Time course for root length in 1RS (2R), 1RS^{RW} (4R+2B), and recombinant lines T-21 and 1B+40 (2R+2B); $N = 10$ plants per genotype and time point (except for T-21 = 7). (c) Root growth rate for the same lines as in (b). (d) Independent experiment with the same lines as in (b) but with the addition of T-18, which carries a large terminal 1BS segment and no duplications (2B); $N = 24$ plants per genotype and time point (except for T-21 = 14 and 1RS = 21). (e) Root growth rate for the same lines as in (d). (b and d) Different letters in the last time point (same color as the curve) indicate significant differences using a Tukey test ($P < .05$)

significant (contrast 1RS vs. combined T-21 and 1B+40, $P = .1534$; Figure 3b). The contrast between 1RS^{RW} and combined T-21 and 1B+40 was highly significant ($P < .001$), and the two individual lines were also significantly different from 1RS^{RW} in individual Tukey tests (Figure 3b). An additional graph of the same results showing root growth rate (mm h^{-1}) as a function of time showed that the shorter roots of 1RS^{RW} were the result of a rapid decrease in root growth around 11.5 d (Figure 3c).

In the second hydroponic experiment, we included the same lines as in the first experiment and added line T-18 as a 1BS control (copy number formula = 2B), for which we completed the four backcrosses into Hahn later. The roots of T-18 were longer than the roots from other genotypes since the initial time we started measuring (9 d), and this was reflected in significant differences with all other lines except for 1B+40 at the end of the experiment (Figure 3d). However, root growth rate in T-18 and 1RS were almost identical for all measurements (Figure 3e), suggesting that the final differences in root length were the results of the early differences in growth. For some unknown reason, roots from all genotypes showed an earlier decrease in growth rate than in the first experiment, which was typical of our previous experiments (Howell et al., 2019).

In spite of the reduced differences among lines, the final root length of 1RS^{RW} was significantly shorter than those from 1RS, T-21, and 1B+40 (Figure 3d). As in the first experiment, the contrast between the control 1RS line and combined T-21 and 1B+40 was not significant ($P = .1958$; Figure 1d), but the two lines carrying the 2B+2R duplication tended to display an earlier decrease in root growth when compared with the combined 1RS and T-18 lines at 17.5 days ($P = .049$; Figure 1e). Taken together, the results in Figure 3 indicate that the addition of the 1BS segment in T-21 and 1B+40 (2R+2B)

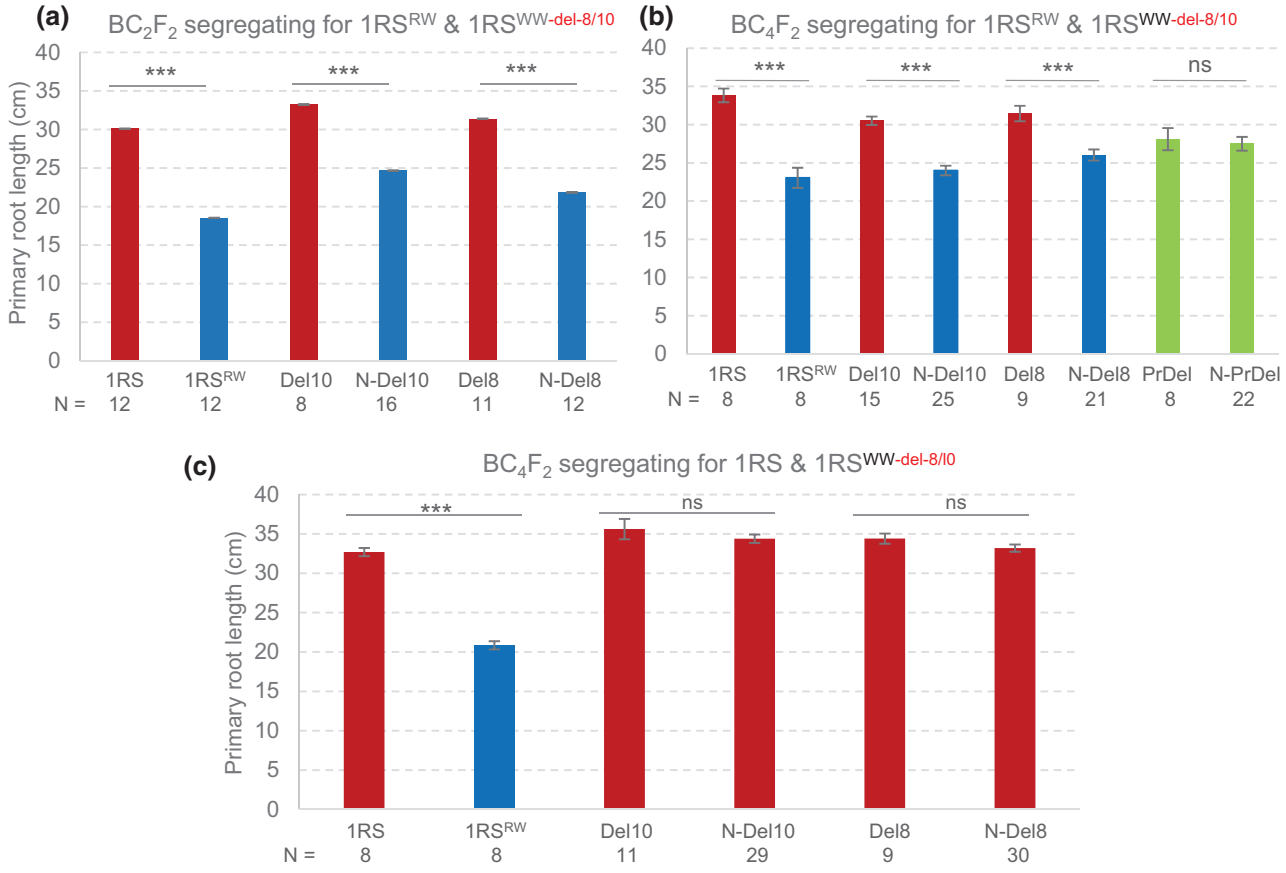

FIGURE 4 Root length of plants segregating for the $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ deletions backcrossed to Hahn-$1RS^{RW}$ and Hahn-1RS. (a) BC_2F_2 population segregating for the two deletions backcrossed to $1RS^{RW}$. (b) BC_4F_2 population segregating for the same two deletions after two additional generations of backcrossing. In this experiment, the $1RS^{WW\text{-}del\text{-}8}$ segregated for the proximal 1BS segment that replaced the orthologous 1RS segment between 16.34-25.38 Mb (PrDel = homozygous wheat allele, N-PrDel = heterozygous and homozygous rye segment combined). (c) BC_4F_2 population segregating for $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ backcrossed into Hahn-1RS; *N* = number of plants genotyped and phenotyped for each genotype. ***Significant at the .001 probability level; ns, nonsignificant at the .05 probability level

has a smaller effect in decreasing root length than the addition of a 1RS copy in heterozygous plants (3R+1B) or two 1RS copies in the $1RS^{RW}$ line (4R+2B) (Figure 3a).

3.5 | Radiation mutants in Hahn-$1RS^{RW}$ showed long root phenotype similar to 1RS

To confirm the effect of the duplicated region on root length and to dissect the candidate gene region, we compared the root lengths of $1RS^{RW}$ with the deletion lines $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ (Figure 2a). We first backcrossed the two deletion lines independently to Hahn-$1RS^{RW}$ and to Hahn-1RS to reduce the level of background mutations and phenotyped the segregating plants for root length after two (BC_2F_2) and four (BC_4F_2) backcrosses (Figure 4). We used the 1BS markers THdw06 located outside the deleted region and marker 1B70E (Supplemental Table S1) located within the deleted 1BS region to identify 1BS deletion homozygotes. We were unable to differentiate between homozygotes or heterozygotes for the nondeleted $1RS^{RW}$ chromosome because of the duplicated 1RS flanking region in $1RS^{RW}$. We refer to these plants as nonhomozygous for the deletion (N-Del).

For the deletion lines crossed to Hahn-$1RS^{RW}$, we performed two hydroponic experiments: one at BC_2F_2 (Figure 4a) and the other one at BC_4F_2 (Figure 4b). Since the results were similar, we performed the statistical analysis combing both experiments and using experiment as block. The roots of the lines homozygous for deletions $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ were significantly longer than the roots of the N-Del sister lines in all four individual comparisons and in the combined analysis ($P < .001$; Figures 4a and 4b). However, the combined analyses showed that the N-Del lines were on average 4 cm longer than the $1RS^{RW}$ recurrent parent ($P < .001$), which is not surprising since the N-Del lines were a mixture of $1RS^{RW}$ homozygotes and heterozygotes. This confirms the importance of the dosage of the duplicated region in the determination of the root length.

For the deletion lines crossed to Hahn-1RS, we detected no significant differences in the root length between the

deletion homozygotes and their segregating BC_4F_2 sister lines carrying at least one standard 1RS chromosome arm (Figure 4c). The same result was observed when the deletion homozygotes $1RS^{WW\text{-}del\text{-}8}$ (31.5 cm) and $1RS^{WW\text{-}del\text{-}10}$ (31.3 cm) from crosses with Hahn-$1RS^{RW}$ were compared with the 1RS control line (31.7 cm), rather than with their corresponding sister lines (Figures 4a and 4b, $P = .70$ combined ANOVA using experiment as block).

It is unlikely that radiation deletions in other chromosomes were the cause of the long roots of $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ because we observed identical effects in the independent backcrosses of the two lines into two different backgrounds. We also failed to find linked deletions in the 1RS chromosome using the exome capture data from both deletion lines. The only other deletion in 1RS was between 16.3 and 25.4 Mb, which is the region replaced by the proximal wheat 1BS segment (RefSeq v1.1, 17.6–26.8 Mb). This proximal wheat segment was already present in the irradiated F_2 plants derived from the cross 1RS × $1RS^{WW}$ (Figure 1).

We have previously shown that the proximal wheat segment has no effect on root length (Howell et al., 2019), and this was confirmed here using 30 BC_4F_2 plants from the progeny of a cross $1RS^{RW}$ × $1RS^{WW\text{-}del\text{-}8}$ segregating for the radiation deletion in the distal wheat segment and the proximal wheat segment. We traced the primary deletion in the progeny using 1BS markers THdw06 (outside deletion) and 1B70E (inside deletion; Supplemental Table S1). For the proximal wheat segment, we used 1BS marker wPt1911 (Howell et al., 2014) and markers 1RS6184179 and o-sec-up/low (secalin) (Shimizu, Nasuda, & Endo, 1997), both located within the orthologous rye region (Supplemental Table S1). A factorial ANOVA for root length showed a highly significant effect for the primary deletion ($P < .001$), no significant differences for the proximal wheat segment ($P > .05$; Figure 4b, green bars), and no significant interaction between the two factors. Taken together, these results support the conclusion that the 4.3–4.9 Mb deletion encompassing adjacent regions of the 1BS and 1RS arms caused the longer roots of $1RS^{WW\text{-}del\text{-}8/10}$ relative to $1RS^{RW}$.

3.6 | Characterization of the candidate genes located within the deletion in the 1BS-1RS border

We analyzed the maximum deleted region in 1BS (1.56 Mb, 8.00–9.56 Mb) in the CS RefSeq v1.1 and identified 38 high confidence genes (*TraesCS1B02G016500*–*TraesCS1B02G020300*). An analysis of the expression profiles of these 38 genes in different tissues and developmental stages in published RNASeq studies that included 89 root samples (Borrill, Ramirez-Gonzalez, & Uauy, 2016) showed that only 18 of these 38 genes were expressed in roots (Figure 5). Since genes that are not expressed in the roots are less likely to affect root development, we excluded such genes from further analysis.

An analysis of the colinearity between the genes located within the deleted region in $1RS^{WW\text{-}del\text{-}8/10}$ revealed that even though the region deleted in $1RS^{AK58}$ (max. 3.3 Mb) is longer than the region deleted in 1BS (max. 1.56 Mb), the deleted 1BS region covers a longer orthologous region. Table 1 shows that most of the deleted genes include both the 1BS and 1RS orthologues, with the exception of 1BS genes *TraesCS1B02G017200* and *TraesCS1B02G017300*, which are inside the 1BS deleted region in $1RS^{WW\text{-}del\text{-}8/10}$, whereas its 1RS orthologues are outside the deletion borders (Table 1, underlined). Since the duplication of the 1RS region showed the strongest effect on the root length and the radiation mutant showed roots of the same length as 1RS, we excluded the last two genes, reducing our prioritized candidate list to 14 high-confidence annotated genes that were deleted both in the 1BS and the duplicated 1RS segments (Table 2).

In Table 2, we summarize the annotation of the 14 genes, which includes four disease resistance genes, three jasmonic acid biosynthetic genes, two small GTP-binding proteins (RAB-like), two chaperone proteins (tubulin-specific chaperone cofactor E-like, and DNAJ), a wall-associated receptor kinase (WAK), a methionine S-methyltransferase, and an E3 ubiquitin-protein ligase CHIP-like. The potential roles of these proteins in the observed phenotypes are included in the Discussion section.

4 | DISCUSSION

4.1 | Evolving hypothesis for the genes affecting the short-root phenotype

Previous field studies demonstrated that cultivar Hahn carrying the standard 1RS.1BL translocation had longer roots, better access to water, and significantly higher grain yields than isogenic Hahn lines carrying the $1RS^{RW}$ chromosome (Howell et al., 2014, 2019). Hydroponic studies confirmed that 2 wk after germination, the roots in Hahn-$1RS^{RW}$ showed a significant reduction in the elongation rate, altered gradients of reactive oxygen species, and the emergence of lateral roots close to the RAM (Howell et al., 2019). This earlier reduction in root growth rates in $1RS^{RW}$ relative to 1RS was also observed in this study, even in experiments that showed variable overall root growth responses (Figures 3c and 3e).

We initially assumed that the 4.8 Mb 1BS segment in the $1RS^{RW}$ chromosome arm was the result of a homologous recombination event between the overlapping 1BS segments of lines T-9 (distal 1BS) and 1B+40 (distal 1RS; Figure 1) (Lukaszewski, 2000) and that, therefore, the 1BS wheat genes have replaced the orthologous 1RS rye genes.

TABLE 2 Candidate genes expressed in the root and deleted in $1RS^{RW\text{-}del\text{-}8/10}$. Numbers inside the table are average transcripts per million using a log 2 scale. The number of RNA Sequencing samples used to calculate the averages (bottom row) are based on the Wheat Expression Browser (https://www.wheat-expression.com/)

	Root			Leaf			Spike	Grain	
Gene RefSeq v1.1	Seed. [a]	Veg. [a]	Repro. [a]	Seed. [a]	Veg. [a]	Repro. [a]	Repro. [a]	Repro. [a]	Annotation, high-confidence genes
TraesCS1B02G017500	0.00	0.10	0.42	0.00	0.00	0.00	0.00	0.00	NB-ARC-LRR disease resistance
TraesCS1B02G017600	0.74	1.59	1.31	0.00	0.00	0.00	0.00	0.00	NB-ARC-LRR disease resistance
TraesCS1B02G017700	2.08	3.38	4.56	4.44	4.04	0.69	4.37	3.45	Defensin
TraesCS1B02G017800	5.10	4.95	5.40	3.61	4.30	4.47	2.77	0.43	Methionine S-methyltransferase
TraesCS1B02G017900	1.06	1.28	1.97	1.30	1.37	1.39	0.40	0.30	E3 ubiquitin-protein ligase CHIP
TraesCS1B02G018100	3.56	0.34	0.00	1.31	0.00	0.00	0.00	4.57	Defensin
TraesCS1B02G018700	3.14	4.25	2.92	0.00	0.00	0.00	0.00	0.00	12-oxophytodienoate reductase
TraesCS1B02G018900	3.56	3.84	2.83	2.45	2.58	0.81	2.03	2.28	Small GTP-binding protein (RAB)
TraesCS1B02G019100	4.91	4.87	4.90	4.23	4.10	4.01	3.73	4.39	Small GTP-binding protein (RAB)
TraesCS1B02G019200	3.81	3.40	3.82	2.44	2.35	2.70	2.20	2.41	Tubulin-specific chaperone cofactor E
TraesCS1B02G019300	2.13	1.54	2.05	5.84	6.14	6.64	4.32	0.00	Chaperone protein DNAJ
TraesCS1B02G019700	2.09	3.25	1.66	0.00	0.00	0.00	0.00	0.00	12-oxophytodienoate reductase
TraesCS1B02G019800	0.91	0.00	0.00	1.69	0.96	0.00	0.00	0.00	12-oxophytodienoate reductase
TraesCS1B02G020200	1.13	0.83	0.00	0.00	0.00	0.00	0.00	0.00	Wall-associated receptor kinase
No. of samples	8	73	8	174	156	151	278	166	–

[a]Seed., seedling stage; veg., vegetative stage; repro., reproductive stage.

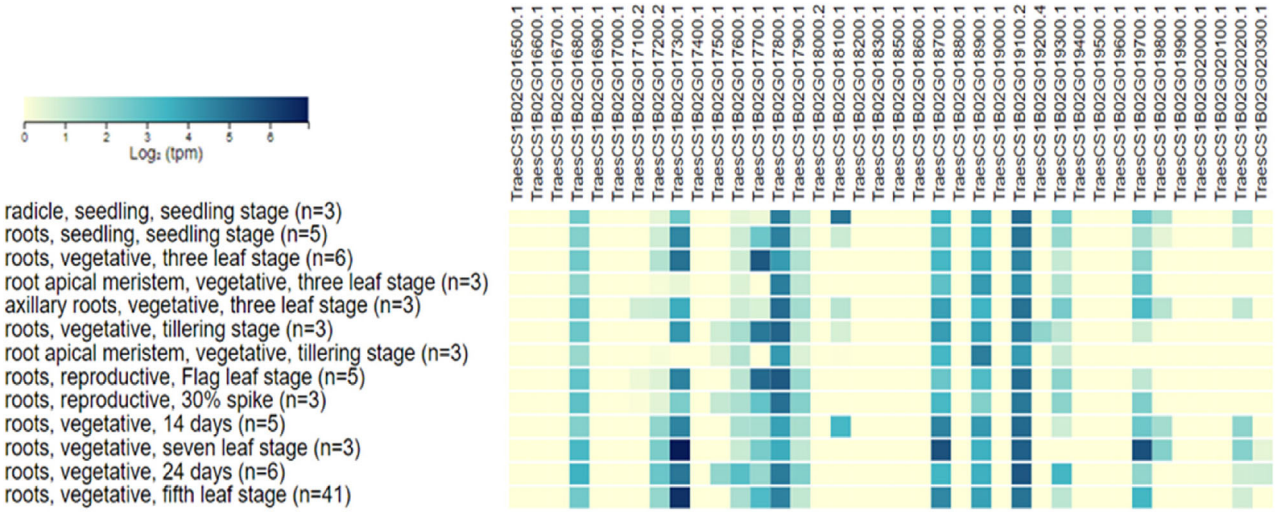

FIGURE 5 Root expression profiles of the 38 high-confidence genes identified in the 1.56 Mb region deleted in chromosome arm 1BS in $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$. Expression data from 89 root samples from 13 different RNA-Sequencing studies compiled by ExpVIP (http://www.wheat-expression.com/, darker blue indicates higher expression)

Given the known positive effect of the 1RS translocation on drought tolerance in wheat, we hypothesized that the lost 1RS genes were the cause of shorter roots in $1RS^{RW}$. However, the exome capture sequencing of 1RS and $1RS^{RW}$ demonstrated that both the 1BS and its orthologous 1RS segment were still present in $1RS^{RW}$, disproving our original hypothesis.

Our second hypothesis was that wheat genes present in the 4.8-Mb 1BS segment inserted in $1RS^{RW}$ could be responsible for the shorter roots. However, the characterization of the Hahn-T-18 line, which carries a 17-Mb distal 1BS segment (including the 4.8 Mb of the 1BS segment in $1RS^{RW}$) and has no identifiable duplications, provided evidence against this hypothesis. The roots of T-18 were slightly longer than those in 1RS at the initiation of the measurements (9 d; Figure 3d) but showed no significant differences in their root growth rates after that day (Figure 3e). When the 1BS segment was combined with the 1RS segment in the Hahn-T-21 and Hahn-1B+40, the roots were significantly longer than the roots of $1RS^{RW}$ and slightly, but not significantly, shorter than the roots in the control 1RS line (Figure 3b). Taken together, these results provided conclusive evidence that the presence of the wheat genes in the 1BS segment alone was not responsible for the short roots $1RS^{RW}$ and disproved our second hypothesis.

Our third, and still current, hypothesis, is that the change in gene dosage generated by the duplications of the 1BS and 1RS colinear regions was responsible for the arrest in the seminal root growth. The lack of differences in root growth rate between T-18 (2B copies) and 1RS (2R copies) between 9 and 28 d suggest that the genes in the 1BS segment are not responsible for the reduced growth rate in $1RS^{RW}$ during the same period (Figure 3e). The 1BS-1RS duplication in T-21 and 1B+40 (2R+2B) resulted only in a minor decrease in growth rate relative to 1RS (2R) and their final root lengths were significantly longer than in $1RS^{RW}$ (Figure 3b and 3d). As T-21 tended to be shorter than 1B+40 in both experiments, we cannot rule out the possibility that their different proximal regions (1RS in T-21 and 1BS in 1B+40; Figure 2) may contribute to modulate the effect of the 2R+2B duplication on root length. These results suggest that adding duplicated 1BS genes has a smaller effect on seminal root growth than adding more copies of the 1RS orthologues. The stronger effect of the 1RS segment was evident in plants heterozygous for the $1RS^{RW}$ chromosome (3R+1B), which showed seminal root length intermediate to that of 1RS and $1RS^{RW}$ (Figure 3a). Based on this result, we hypothesize that the duplication of the 1RS region in $1RS^{RW}$ (4R+2B) is the main driver for shorter roots in this line, but we do not entirely discard the idea that the genes in the 1BS segment may also contribute to the reduced root growth when combined with additional 1RS orthologues.

The dosage hypothesis was reinforced by the hydroponic experiments with the radiation-mutants $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ backcrossed independently to both to Hahn-$1RS^{RW}$ and Hahn-1RS. In the hydroponic experiments using the backcross lines segregating for the deletions and $1RS^{RW}$, the roots of the deletion lines were significantly longer than those of the sister lines carrying at least one $1RS^{RW}$ chromosome (Figures 4a and 4b). By contrast, in the lines segregating for the deletions and the 1RS chromosome, we observed no significant differences in root length between the homozygous deletions and their sister lines carrying at least one 1RS chromosome (Figure 4c).

The four consecutive backcrosses of $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ into $1RS^{RW}$ and 1RS minimized the chances of a possibly confounding effect of independent deletions

in other chromosomes of the radiation mutants. However, they did not rule out the possibility of a confounding effect of a linked deletion in 1RS. Using the exome capture, we did find a linked missing 1RS region corresponding to the orthologous rye region replaced by the proximal wheat segment in homozygotes for the $1RS^{WW}$ chromosome. We have previously shown that the proximal wheat segment has no effect on root length (Howell et al., 2019) and confirmed this result in the hydroponic experiments presented in this study (Figure 4b).

The exome capture data also allowed us to determine the length of the 1RS deleted segment in the deletion lines (both lines carry the same deletion) and to establish that the 1BS and 1RS deletions include mostly orthologous genes (Table 1; Supplemental Figure S3). Therefore, the homozygous $1RS^{WW\text{-}del\text{-}8}$ and $1RS^{WW\text{-}del\text{-}10}$ lines are expected to lose two gene copies in 1BS and two in 1RS, changing the gene dosage from 4R+2B to 2R. This hypothesis explains the identical seminal root size observed in the 1RS (2R) and the homozygous deletion lines (Figure 4c).

One limitation of the exome capture assays is that they are closed systems and some genes are not included, which resulted in annotated genes with no reads. We eliminated those genes for the analysis used to delimit the borders of the 1RS–1BS recombination events or of the duplicated 1RS region (Supplemental Figures S1, S2, and S5). This likely resulted in a slight overestimate of the size of the candidate gene regions and the number of potential candidate genes.

4.2 | Candidate genes for the short-root phenotype

Once we established conservative borders of the 1BS and 1RS deleted regions in $1RS^{RW\text{-}del\text{-}8/10}$, we considered all the annotated genes in these regions as candidates regardless of their presence in the exome capture. The $1RS^{AK58}$ genome is very close to the 1RS present in our lines, so it probably provides a good representation of the rye candidate gene region. However, the CS RefSeq 1.1 used as 1BS reference is not identical to the 1BS Pavon segment, and therefore, we cannot rule out the possibility of genes present in Pavon that are not present in the wheat reference.

Since the deletion mutants showed similar root length as the 1RS line, we decided to focus on the 14 genes expressed in roots that were deleted in both the 1RS duplication and the adjacent and orthologous 1BS insertion (Table 2). Although the annotated functions of these genes based on conserved domains and homology will require further experimental validation, the list is useful to summarize their inferred functions and to provide a preliminary idea of potential candidate genes.

The first group includes four genes annotated as defense genes, a function that is likely not closely related with the observed phenotypes. This group includes *TraesCS1B02G017500* and *TraesCS1B02G0017600* (48% similar), which encode proteins with NB-ARC and LRR domains characteristic of plant disease-resistance proteins involved in pathogen recognition and activation of immune responses. It also includes *TraesCS1B02G017700* (77 amino acids) and *TraesCS1B02G0018100* (81 amino acids, 83% similar), which are both annotated as defensins, a family of small plant antimicrobial peptides that serve to defend plants against pathogens.

A second group includes three genes annotated as having enzymatic or housekeeping functions, which may not be compatible with the developmental nature of the observed changes in the roots of $1RS^{RW}$. The first gene in this group, *TraesCS1B02G017800*, encodes a methionine S-methyltransferase that has been implicated in the volatilization of selenium (Tagmount, Berken, & Terry, 2002) and in the biosynthesis of S-methylmethionine, a compound that is important in the transport of sulfur (Bourgis et al., 1999). The last two genes in this group encode proteins with chaperon functions. *TraesCS1B02G019200* is a tubulin-folding cofactor E (TBCE, based on similarity with *Arabidopsis* AT1G71440) involved in the second step of the tubulin folding pathway. *TraesCS1B02G019300* encodes a chaperone protein DnaJ, which stimulates the heat-shock protein Hsp70's ATPase activity, stabilizing its interaction with client proteins. These chaperon proteins play important roles under plant stress (Rana, Iqbal, Wattoo, Khan, & Zhang, 2018) but are unlikely to play an important role in the phenotypic differences we observed under optimal hydroponic conditions.

The third group includes genes involved in regulatory processes or in cell growth or division, processes more likely to be involved in the observed developmental changes in root growth (Howell et al., 2019). *TraesCS1B02G017900* encodes an E3 ubiquitin-protein ligase CHIP-like protein that ubiquinate heat shock misfolded client proteins, targeting them for proteasomal degradation. Since E3 ubiquitin-protein ligases can ubiquitinate and regulate multiple targets, we could not rule it out as a potential candidate gene. We also included in this group the genes *TraesCS1B02G018900* and *TraesCS1B02G0019100*, which encode 64% similar small GTP-binding proteins from the RAB family. These conserved proteins serve as molecular switches in signal transduction and play important roles in intracellular membrane trafficking, cross-talk with plant hormones and regulation of organogenesis, polar growth, and cell division (Ma, 2007), all functions that seem relevant to the observed differences in root development. *TraesCS1B02G018700*, *TraesCS1B02G019700*, and *TraesCS1B02G019800* encode 12-oxophytodienoate reductase-like proteins involved in the biosynthesis of jasmonic acid. Since hormones can affect

multiple developmental traits, these are also strong candidate genes. Finally, *TraesCS1B02G020200* encodes a wall-associated receptor kinase (WAK). These serine–threonine kinases are involved in signaling and cell expansion, making it an interesting candidate for the differences in root length observed in $1RS^{RW}$.

5 | CONCLUSIONS

This study demonstrates the value of alien introgressions in the dissection of important agronomic traits in wheat but it is also a cautionary tale of the complex rearrangements that can be generated by hidden structural variation. Fortunately, powerful genomic tools are now available to understand these chromosome rearrangements, which are critical to interpret correctly the phenotypic results. In this particular case, although we observed small differences in root length between the wheat and rye alleles, the most dramatic effects on root development were the result of changes in gene dosage originated by segmental chromosome duplications. We confirmed the importance of the changes in dosage using a radiation mutant in which a large deletion restored the normal gene copy number and the production of long roots. This deletion mutant, together with publicly available RNASeq data, was critical to delimit a set of 14 candidate genes. Given the pleiotropic effects of this duplication (e.g. RAM growth arrest, region of emergence of lateral roots, and altered gradients of reactive oxygen species), we currently favor candidate genes that can have multiple pleiotropic effects. We have initiated RNASeq experiments to provide additional information to prioritize candidate genes for functional validation using transgenic approaches. We hope that the identification of the genes that cause the drastic changes observed in root development will also help us to find the natural variants that helped wheat to adapt to multiple soil types and become a globally important crop.

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DATA AVAILABILITY STATEMENT

All the exome capture data has been deposited in GenBank with BioProject numbers PRJNA663626 (Hahn), PRJNA663630 (Hahn-$1RS^{RW}$), PRJNA663627 (Hahn-$1RS^{RW}$ EMS mutant RW_M4_43_11), PRJNA663628 (Hahn-$1RS^{RW}$ EMS mutant RW_M4_47_12), PRJNA663632 (Radiation deletion mutant 8 of Hahn-$1RS^{WW}$), PRJNA663634 (Radiation deletion mutant 10 of Hahn-$1RS^{WW}$), PRJNA663635 (Pavon76-1B+37), PRJNA663636 (Pavon76 1B+40), PRJNA663637 (Pavon76-T-9), PRJNA663640 (Pavon76-T-18), and PRJNA663641 (Pavon76-T-21). Genetic materials are available at the National Small Grain Collection under accession numbers PI 672839 (Hahn-$1RS^{RW}$), PI 672838 (Hahn-$1RS^{WR}$), and PI 672837 (Hahn-$1RS^{WW}$). Other stocks are available from the authors upon request.

AUTHOR CONTRIBUTIONS STATEMENT

Gilad Gabay: experimental, generation of materials, data analyses, wrote first draft of the manuscript and review manuscript. Junli Zhang: experimental, generation of materials, data analysis, review manuscript. German Federico Burguener: data analysis, review manuscript. Tyson Howell: experimental, generation of materials, data analysis, review manuscript. Hanchao Wang: experimental, review manuscript. Tzion Fahima: generation of materials, review manuscript. Adam Lukaszewski: generation of materials, review manuscript. Jorge Moriconi: experimental, data analysis, review manuscript. Guillermo E. Santa Maria: experimental, data analysis, supervision, review manuscript. Jorge Dubcovsky: project conception and direction, funding, supervision, data analysis, review manuscript and wrote final version.

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflicts of interest.

ORCID

Jorge Dubcovsky https://orcid.org/0000-0002-7571-4345

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SUPPORTING INFORMATION

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