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ORIGINAL ARTICLE

Distribution and haplotype diversity of *WKS* resistance genes in wild emmer wheat natural populations

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Abstract

Key message **The wheat stripe rust resistance gene *Yr36* (*WKS1*) with a unique kinase-START domain architecture is highly conserved in wild emmer wheat natural populations.**

Abstract Wild emmer wheat (*Triticum dicoccoides*) populations have developed various resistance strategies against the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* (*Pst*). The wild emmer gene, *Yr36* (*WKS1*), which confers partial resistance to a broad spectrum of *Pst* races, is composed of a kinase and a START lipid-binding domain, a unique gene architecture found only in the Triticeae tribe. The analysis of 435 wild emmer accessions from a broad range of natural habitats revealed that *WKS1* and its paralogue *WKS2* are present only in the southern distribution range of wild emmer in the Fertile Crescent, supporting the idea that wheat domestication occurred in the northern populations. An analysis of full-length *WKS1* sequence from 54 accessions identified 15 different haplotypes and very low-nucleotide diversity ($\pi = 0.00019$). The high level of *WKS1* sequence conservation among wild emmer populations is in contrast to the high level of diversity previously observed in NB-LRR genes (e.g., *Lr10* and *Pm3*). This phenomenon may reflect the different resistance mechanisms and different evolutionary pathways that shaped these genes, and may shed light on the evolution of genes that confer partial resistance to stripe rust. Only five *WKS1* coding sequence haplotypes were revealed among all tested accessions, encoding four different putative WKS1 proteins (designated P0, P1, P2, and P3). Infection tests showed that P0, P1, and P3 haplotypes display a resistance response, while P2 displayed a susceptible response. These results show that the WKS1 proteins (P0, P1, and P3) can be useful to improve wheat resistance to stripe rust.

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Introduction

Plant diseases are one of the major threats to crop production. Stripe rust (*Puccinia striiformis* f. sp. *tritici*), leaf rust (*Puccinia triticina*), and stem rust (*Puccinia graminis* f. sp. *tritici*) are among the most damaging fungal diseases of wheat. Plant resistance to these pathogens, which is conferred by a series of disease resistance genes (McIntosh et al. 2008), has provided an economical and sustainable approach to control these diseases.

Generally, plant disease resistance can be classified as qualitative resistance that is conferred by a single resistance gene often known as race-specific resistance (henceforth, R

genes), or as quantitative resistance mediated by multiple quantitative trait loci (QTL) (Kou and Wang 2010). Race-specific resistance usually provides a temporary solution since fungal populations rapidly evolve to overcome this type of resistance. In contrast, quantitative resistance (QR) offers a broad-spectrum resistance that is partial and, in some cases, has proven to be more durable than the resistance conferred by single R genes (Krattinger et al. 2009).

R genes often encode proteins with nucleotide-binding (NB) and leucine-rich repeat (LRR) domains (Keller et al. 2005; Periyannan et al. 2013; Saintenac et al. 2013). Previous studies indicated that plant R genes tend to show high levels of sequence diversity as compared with other genes not associated with pathogen resistance. Furthermore, R gene diversity is associated with pathogen specificity and maintained by diversifying or balancing selection (Bergelson et al. 2001; Kuang et al. 2008; Mauricio et al. 2003; Mondragon-Palomino and Gaut 2005; Sela et al. 2011; Wicker et al. 2007; Yahiaoui et al. 2009).

Molecular evolution and genetic diversity studies of QR genes conferring broad-spectrum resistance have been less reported. The characterization of nucleotide diversity of the leaf rust QR gene *Lr34* revealed relatively low-sequence diversity. In the wheat gene pool, two predominant *Lr34* alleles (*Lr34res-D* and *Lr34sus-D*) were found that differ by only two critical codons in exon 11 and exon 12 (Dakouri et al. 2010; Krattinger et al. 2009, 2011; Lagudah et al. 2009; McCallum et al. 2012).

The positional cloning of the wheat stripe rust QR gene *Yr36* provided us with a unique opportunity to understand the potential of its encoded protein and the mechanism of broad-spectrum resistance (Fu et al. 2009; Gou et al. 2015). The *Yr36* gene is derived from wild emmer wheat, *Triticum turgidum* ssp. *dicoccoides* (Korn.) Thell (*T. dicoccoides*, hereafter) ($2n = 4\times = 28$, BBAA). This gene confers partial and a broad-spectrum stripe rust resistance at relatively high temperatures (above 25 °C) (Fu et al. 2009). A recent report indicated that *Yr36* is effective at relatively cooler temperatures (below 18 °C) to UK isolates of stripe rust (Segovia et al. 2014). *Yr36* is located on chromosome 6BS and was designated as wheat kinase-START (*WKS1*). It encodes a protein with a unique combination of a functional kinase and lipid binding START domain (Gou et al. 2015) that was found only in the Triticeae tribe. Functional analysis of a set of TILLING (targeting induced local lesion in genome) mutants demonstrated that both the kinase and the START domains are necessary for conferring disease resistance responses. Further transformation experiments of susceptible wheat varieties confirmed that *WKS1* alone was sufficient to improve resistance to *P. striiformis* (Fu et al. 2009; Gou et al. 2015). *WKS1* confers resistance by inducing necrosis in the area where the pathogen has invaded, a process that is mediated by WKS1 phosphorylation of the thylakoid associated ascorbate peroxidase (tAPX). Phosphorylation of tAPX reduces its peroxidase activity, resulting in an increase in reactive oxygen species and cell death (Gou et al. 2015). Interestingly, *WKS1* gene was detected in only ~24 % of the wild emmer wheat accessions tested by Fu et al. (2009) and was not detected in any of the tested bread or durum wheat varieties. These findings suggest that *WKS1* was not present in the initial domesticated forms of emmer wheat.

The *WKS2* gene is a paralog of *WKS1* and was found to be adjacent to *WKS1*. The protein encoded by *WKS2* is 86 % identical to *WKS1* at the protein level. The major difference between *WKS1* and *WKS2* in tetraploid wheat is an insertion of a 3.5 kb transposable element (TE) in the tenth intron of *WKS2*. Functional analysis of a set of TILLING mutants demonstrated that *WKS2*, which contains the transposon insertion, is not required for conferring resistance to *P. striiformis* f. sp. *tritici* (*Pst*) (Fu et al. 2009).

Wild emmer wheat, *T. dicoccoides* (Korn.) Thell, is the tetraploid progenitor of cultivated bread wheat (McFadden and Sears 1946). The natural habitats of *T. dicoccoides* are located in the Middle East Fertile Crescent. Natural wild emmer wheat populations are dispersed over a wide range of habitats in Israel, Jordan, Syria, Lebanon, Turkey, Iran and Iraq, where they are challenged by the stripe rust pathogen. During the coevolution of wild emmer and stripe rust, the wheat populations developed various resistance strategies. Numerous studies reported that many *T. dicoccoides* accessions originating in Israel were highly resistant to wheat stripe rust isolates at seedling and adult stages (Cheng et al. 2010; Fahima et al. 1998; Gerechter-Amitai and Stubbs 1970; Nevo et al. 1986, 2002; The et al. 1993; Van Silfhout et al. 1989). Several genotypes showed temperature-dependent resistance usually activated at high temperature (Gerechter-Amitai et al. 1984; Gerechter-Amitai and van Silfhout 1989).

The objectives of the present study were: (1) to study the distribution of *WKS* genes among the natural *T. dicoccoides* populations and search for association with ecogeographic factors at the original habitats; (2) to evaluate the level of sequence diversity of *WKS1* gene in *T. dicoccoides* natural populations; (3) to characterize the stripe rust resistance in a wide collection of Israeli *T. dicoccoides* accessions, which carry *WKS1*; (4) to compare the nucleotide diversity patterns of race-specific (NB-LRR) versus race non-specific (*WKS1*) R genes.

Materials and methods

Plant material

A total of 383 accessions of *T. dicoccoides* collected from 125 collection sites representing the ecogeographic

distribution of *T. dicoccoides* in Israel and its vicinity, and a total of 52 accessions collected from natural populations in Jordan, Lebanon, Syria, Iran, and Turkey were used in the current study. The list of accessions and their collection sites are described in Supplementary file 1.

Analysis of presence/absence polymorphism in *WKS1* and *WKS2*

Wheat DNA was extracted from 10-day-old seedlings using the ArchivePure DNA Cell/Tissue Kit (5 Prime, Gaithersburg, MD, USA). Presence/absence of both *WKS1* and *WKS2* genes was analyzed using PCR amplification with gene-specific primers of *WKS1* and *WKS2* (Supplementary file 2). PCR was performed using the Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) in 15 µl reaction volume containing 1× PCR buffer, 80 ng of genomic DNA, 300 nM of each primer, 200 µM of each dNTP, and 0.5 U of Dream Taq™ DNA Ploymerase (Thermo Scientific, Waltham, MA, USA). Touchdown PCR amplification was performed as follows: 3 min at 95 °C; ten cycles of 30 s at 95 °C, annealing Tm as described in Supplementary file 2 (decreasing by 0.5 °C per cycle) for 30 s, and 1 min at 72 °C followed by 25 cycles of 30 s at 95 °C, annealing temperature at constant (Tm-5) °C for 30 s, and 1 min at 72 °C; and a final extension of 7 min at 72 °C. The amplified PCR products were visualized by 1.5 % agarose gel electrophoresis, followed by staining with GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Sequence analysis of *WKS1*

The whole *WKS1* gene region of over 7 kb was amplified from DNA samples of 54 wild emmer genotypes using seven gene-specific primer pairs (Supplementary file 3), designed based on the NCBI GenBank accession EU835198 (Fu et al. 2009). PCR products were extracted from agarose gels using Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA, USA) and cloned into pJET1.2/blunt vector using the CloneJET PCR cloning kit (Thermo Scientific). Samples were sequenced using Big Dye Terminator chemistry on ABI 3130xl Genetic Analyzer (Applied Biosystems). Alternatively, the amplification fragments were purified using Exo-SAP IT (USB, Cleveland, OH, USA) and used for direct sequencing. The SeqMan program (DNAstar, Madison, WI, USA) was used to assemble sequence reads. The obtained sequences were then aligned using Clustal X (Larkin et al. 2007) and manually corrected using BioEdit (Hall 2007). Each SNP was visually checked on the chromatogram to ensure its quality.

DnaSP v5 (Librado and Rozas 2009) was used to perform comparative analysis of *WKS1* sequence alignments, to compute nucleotide diversity (π) as described by Nei and Li (1979), and to test for deviation from neutrality of the haplotypes estimated by Tajima's *D* (Tajima 1989), and Fu and Li's *D* and *F* tests (Fu and Li 1993).

Stripe rust resistance response

Forty-seven Israeli *T. dicoccoides* accessions harboring different *WKS1* haplotypes were tested for their response to stripe rust infection. The highly virulent *Pst* isolate 5006 that belongs to race 38E134 (virulent on *Yr2*, *Yr6*, *Yr7*, *Yr9*, *Yr22*, *Yr23*, *YrSD*, and *YrHVII*; Cheng et al. 2010), kindly provided by Dr. Jacob Manisterski (Tel Aviv University, Israel), was used to inoculate the plants at the fourth leaf stage. Urediniospores used for inoculation of leaf tissue were first suspended in Soltrol® 170 mineral oil (Chevron Phillips Chemical Company) and then sprayed using TLC spray tube (Kontex, Germany). Inoculated plants were placed in a dew chamber (100 % humidity) at 10 °C for 16 h in the dark followed by 8 h of light. Plants were evaluated in two groups for stripe rust resistance under two temperature regimes: (1) growth chamber with 10 °C during the dark period (12 h) and 25 °C during the light period (12 h) (referred as 10/25 °C temperature regime); (2) 10 °C during the dark period (12 h) and at 15 °C during the light period (12 h) (referred as 10/15 °C temperature regime). The resistant isogenic recombinant substitution line RSL65, which carries the functional *WKS1* gene in a *T. durum* cv. Langdon (LDN) genetic background (Fu et al. 2009), was used as a positive control line for the presence of *Yr36*-like QR phenotype. The susceptible 'Bobwhite' (hexaploid) and LDN (tetraploid) cultivars were used as control lines for lack of resistance to *Pst* isolate 5006. Disease severity was evaluated and characterized 14–21 days after inoculation using a 0–9 scale of infection type (IT) (Line and Qayoum 1992).

Correlation analysis of climatic variables with the frequency of *WKS1* gene

A total of 383 accessions were collected from 125 collection sites in Israel (on average 3.06 accessions/per site). Sites that had at least one accession with *WKS* positive signal will be referred hereafter as "positive sites". Forty-one sites with at least four accessions were used for correlation analysis of climatic variables with the frequency of *WKS1* gene. Accessions from the Hermon region were excluded from the correlation analysis because of the low temperatures and presence of other stripe rust qualitative resistance genes that mask *WKS1* quantitative resistance response. Climatic data for each collection site was obtained from worldclim (http://www.worldclim.org) that contained monthly means, maximum and minimum of temperatures of the growth season months (December–April), as well as summaries

of temperatures and precipitation (bioclim, http://www.worldclim.org/bioclim; Supplementary file 4). Evaporation and number of rainy days were obtained from the Israeli Meteorological Service (Noam Halfon, personal communication). The frequency of *WKS1* in each site was tested for correlation with the climatic variables using Spearman correlation (SPSS program; SPSS Inc., Chicago, USA).

Results

Geographical distribution of *WKS* genes and association with climatic factors

To study the occurrence of *WKS* genes (*WKS1* and *WKS2*) in *T. dicoccoides* populations, we screened a total of 435 accessions of *T. dicoccoides*. The accessions were first analyzed using three pairs of gene-specific primers for each *WKS* gene (Fu et al. 2009). Accessions showing successful amplification of the expected fragment size, with at least two primer sets, for each gene separately, were considered as *WKS* positive. Moreover, the *WKS* positive accessions were further screened using two pairs of transposable element-specific primers in the tenth intron of *WKS2* for presence of the retrotransposable element.

The obtained results showed that 62 % of the accessions (271 out of 435) were positive for both *WKS* genes, while 164 (38 %) showed no amplification for either of the *WKS* genes (Supplementary file 1). Interestingly, none of the accessions contained only one of the *WKS* duplicated genes. All of the *WKS* positive accessions had the retrotransposable element insertion in the tenth intron of *WKS2*. In total, 109 out of 125 (87 %) collection sites in Israel and its vicinity included at least one *WKS* positive *T. dicoccoides* accession (Fig. 1a), whereas all of the 28 accessions from Turkey and Iran populations were *WKS* negative. In addition, the *WKS* genes were detected in some of the accessions form Jordan, Syria, and Lebanon populations. Among them, *WKS* genes were present in 80 % (eight out of ten) of *T. dicoccoides* accessions from Syria, 43 % (three out of seven) of accessions from Jordan, while only 14 % (one out of seven) accessions from Lebanon (Supplementary file 1).

The correlations between *WKS1* frequency and the climatic variables were, in general, positive for precipitation variables and negative for temperatures and evaporation variables (Table 1). The correlations of maximum temperatures for all months were significant ($r = -0.31$ to -0.37; $p < 0.05$), as well as the correlation with precipitation in December ($r = 0.31$; $p = 0.049$).

Plotting the presence and absence of *WKS1* sites on the map of Israel shows that *WKS1* is mostly present in the center of wild emmer distribution, the Golan Heights and the Galilee, while it is less abundant near Mt. Hermon and the Judea Mountains (Fig. 1).

Sequence diversity of *WKS1* gene

The sequence encompassing the entire *WKS1* gene was amplified, using overlapping gene-specific primers, in 47 *T. dicoccoides* accessions from 36 collection sites representing the core distribution area of *WKS1* gene in Israel and seven *WKS1* positive accessions from Lebanon, Jordan, and Syria (Supplementary file 1). The 54 full-length *WKS1* sequences were aligned against the reference sequence of the functional *WKS1* from RSL65 (Fu et al. 2009; GenBank EU835199). The size of the sequenced alleles ranged from 7287 to 7288 bp in length, comprising 11 exons and 10 introns. Sequence alignment with the functional *WKS1* indicated that while the whole region of *WKS1* is highly conserved, variable sites were detected in exon 1, intron 1, exon 2, exon 3, intron 10, and exon 11 (Table 2). In the coding parts of *WKS1*, only four SNP sites were identified among the 54 *T. dicoccoides* accessions, which represent 43 different natural habitats. Three of these SNPs are predicted to result in amino acid changes (Table 2): (1) SNP G941A (protein substitution R314H) lies in exon 1 in the kinase domain of *WKS1*; (2) SNP T2598C (W349R) lies in exon 2 between the kinase and the START domains of *WKS1*; and (3) SNP G3738A (G373E) lies in exon 3 between the kinase and the START domains of *WKS1*. SNP C7257T located on exon 11 is not predicted to change the amino acid sequence. SNP G941A was detected in only one accession and SNP T2598C in two accessions, while SNP G3738A in exon 3 was identified in all of the 54 accessions tested in the current study. Three SNPs (SNP1194, SNP1322, and SNP2320), located in a poly-A region of intron 1, were observed in multiple accessions: SNP1194 differs from the functional *WKS1* by the insertion of one adenosine, and was found in 37 % of the accessions. SNP T1322A was identified in the majority (76 %) of the accessions, and SNP T2320A was identified in 44 % of the accessions (Table 2).

A comparative analysis revealed low levels of nucleotide diversity in *WKS1* (Table 3); the average number of nucleotide difference (K) is 1.41, the overall nucleotide diversity (π) is 0.00019, and the overall nucleotide polymorphism (θ_w) is 0.00027. The nucleotide polymorphism level in the intron region of *WKS1* was twofold higher than the level observed in the coding region ($\pi = 0.00022$ vs $\pi = 0.00011$), while the first intron of *WKS1* showed three times higher sequence diversity ($\pi = 0.00064$) than the average *WKS1* intron diversity ($\pi = 0.00022$).

We also evaluated the *T. dicoccoides* genetic variability for *WKS1* gene region, using the Tajima (Tajima 1989) and the Fu and Li Neutrality test (Fu and Li 1993). The purpose

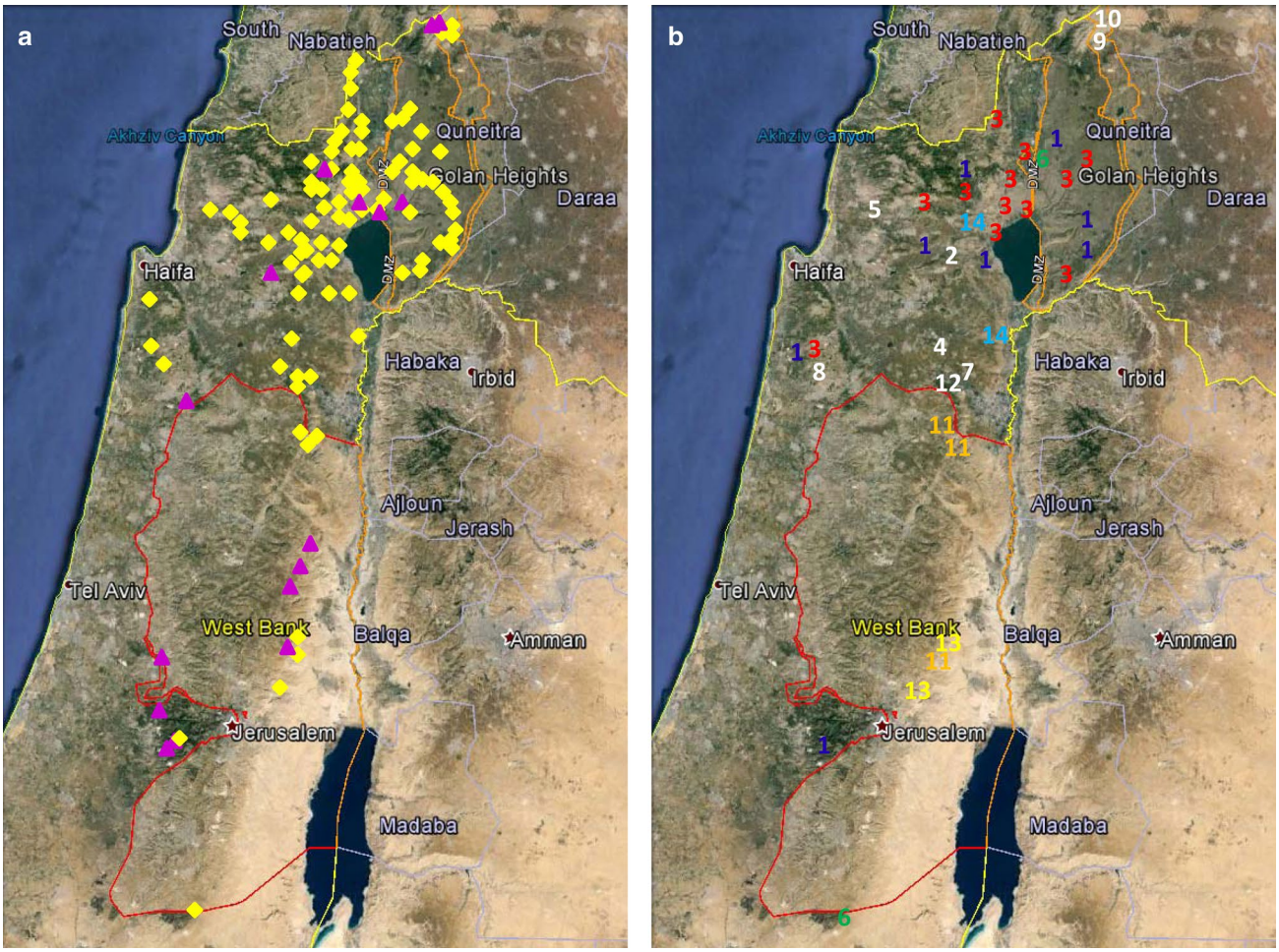

Fig. 1 Geographic distribution of *WKS* genes in Israel and its vicinity. **a** Geographic location of 125 collection sites representing the *T. dicoccoides* range of distribution in Israel and its vicinity. *Squares* indicate sites positive for both *WKS* genes; *triangles* indicate sites negative for both *WKS* genes. **b** The distribution of 14 haplotypes of *WKS1* in Israel and vicinity; *different numbers* indicate different *WKS1* genomic DNA haplotypes; *numbers with white color* indicate haplotypes that were represented by only a single accession

of these tests was to determine if the target DNA sequence conformed to the neutrality model of evolution. Tajima's *D*, Fu and Li's *D*, and Fu and Li's *F* values were calculated to estimate the deviation from the neutral model expectation. In our tests, the Tajima's *D* and Fu and Li's *D* and *F* values for the entire genomic region of *WKS1* sequence were negative (Table 3), a tendency that suggests that this gene may have been shaped by a purifying selection. However, all neutrality tests were not significant ($P < 0.05$), therefore, this observation should be considered as a preliminary trend that needs further validation.

Haplotype diversity

Analysis of the full-length sequence of *WKS1* in 54 *T. dicoccoides* accessions, identified 13 SNP sites defining 15 haplotypes (accessions KT834954 to KT834968) with a haplotype diversity of Hd = 0.814 (Table 4). The published *WKS1* sequence from RSL65 was defined as Hap 0. Fourteen haplotypes (Haps 1-14) were found among 47 accessions from Israel (Supplementary file 5; Fig. 1b), while four haplotypes were detected in seven accessions from Jordan (Haps 1, 8 and 15), Lebanon (Hap 13), and Syria (Haps 1 and 13). In Israel and its vicinity, more than half of the *WKS1* sequences (30 out of 47; 64 %) belonged to only three haplotypes (Hap1, Hap3, and Hap11). Seven haplotypes, represented by a single accession each, were detected only in the northern populations of Israel (Fig. 1b). The highest haplotype diversity of *WKS1* was found in the northern populations where the most frequent haplotype, Hap3, was observed, while Hap 13 was only identified in the southern populations of Israel (Fig. 1b).

Table 1 Spearman correlations of climatic variables against the frequency of *WKS1* in each collection site

Climatic variable[a]	*r*	*p*
Tmax3	−0.37	0.019
Tmax1	−0.35	0.026
Tmax4	−0.35	0.026
Tmax2	−0.34	0.031
Prec12	0.31	0.049
Tmax12	−0.31	0.050
Tmean3	−0.30	0.056
Tmean1	−0.28	0.073
Bio12	0.28	0.076
Prec3	0.28	0.080
Tmean4	−0.27	0.084
Prec2	0.27	0.092
Tmean2	−0.26	0.095
Bio11	−0.26	0.095
Bio16	0.26	0.100
Prec1	0.26	0.100
EW	−0.21	0.186
Tmean12	−0.20	0.200
Tmin1	−0.18	0.268
Bio6	−0.18	0.268
Tmin2	−0.16	0.311
Tmin4	−0.16	0.318
Tmin3	−0.15	0.353
Tmin12	−0.10	0.522
ES	−0.08	0.601

Collection sites ($N = 41$) with at least four accessions were used for the correlation analysis

Tmax maximum temperature of the month, *Tmin* minimum temperature of the month, *Tmean* mean temperature of the month, *Prec* precipitation of the month, *Bio* climatic variables from bioclim, *Bio6* min temperature of the coldest month, *Bio11* mean temperature of coldest quarter, *Bio12* annual precipitation, *Bio16* precipitation of wettest quarter, *EW* evaporation in winter, *ES* evaporation in spring, *r* correlation coefficient, *P* P values

[a] Variables are ordered by the level of correlation. The numbers 12, 1, 2, 3, and 4 that accompany the climatic variables are referring to the months of December, January, February, March, and April, respectively

Analysis of the *WKS1* coding sequences (CDS) of the 54 accessions revealed a total of only four CDS haplotypes (Fig. 2). The most frequent haplotype, CDS_Hap1, was identified in 49 out of 54 accessions, while CDS_Hap2, 3, and 4 were identified in only one or two accessions each. The four CDS_Haps of *WKS1* were detected in accessions from Israel and its vicinity, while all accessions from Lebanon, Jordan, and Syria belonged to CDS_Hap1. When comparing the current DNA coding sequences with the published WKS1 protein from RSL65 (Fu et al. 2009; P0, hereafter), three new types of WKS1 proteins were predicted (Fig. 2; Supplementary file 5): (1) CDS_Hap1 and 4 encode the most frequent type of protein, designated here as WKS1_P1, that has one amino acid change relative to WKS1_P0 (G373E); (2) Haplotype CDS_Hap2, detected in only one genotype, encodes a protein that carries two amino acid changes relative to WKS1_P0 (R314H and G373E) and was designated here as WKS1_P2; (3) CDS_Hap3 encodes a protein designated here as WKS1_P3, also harboring two amino acid changes (W349R and G373E). Among the three changes of amino acids, R314H is positioned inside the kinase domain of the *WKS1* protein, while W349R and G373E are located on the inter-domain region between the kinase and the START domains of the *WKS1* protein (Fig. 2). We did not identify any amino acid changes in the START domain of *WKS1* in any of the 54 sequenced *T. dicoccoides* accessions.

Temperature-dependent stripe rust resistance in *T. dicoccoides* accessions that carry *WKS1*

The functional *WKS1* (GenBank EU835199), found in RSL65, was derived from an Israeli *T. dicoccoides* accession FA15-3, which showed partial resistance to stripe rust of wheat (Fu et al. 2009; Uauy et al. 2005). To test if any of the newly identified *WKS1* alleles confer resistance reactions similar to the original *WKS1* (WKS1_P0 protein), a leaf infection test at the fourth leaf stage was performed in 47 *T. dicoccoides* accessions carrying *WKS1* using Israeli *Pst* isolate 5006 (race 38E134). The differential reactions to stripe rust infection are summarized in Supplementary file 5. The initial testing at 10/25 °C showed that 40 accessions with WKS1_P1 or WKS1_P3 protein revealed a partial resistance (QR) response (IT 2–4; in a 0–9 scale, Line and Qayoum 1992), five (11 %) accessions (WKS1_P1 protein) showed a hypersensitive response (HR) (IT 1) and one (2 %) accession (WKS1_P1 protein) showed an immune response (IT 0) with no visible infection. One accession with haplotype CDS_Hap2 encoding WKS1_P2 (SNP941 G-A, R314H) was completely susceptible (IT 9) to the *Pst* pathogen (Fig. 3). In our test, the phenotype of the positive control line RSL65, carrying a functional allele of *WKS1*, showed an IT of 3 or 4, with necrotic blotches and trace to light sporulation, as can be seen in Fig. 3.

The same set of 47 *T. dicoccoides* accessions were further evaluated at 10/15 °C temperature cycle for resistance to stripe rust. The 40 accessions and positive control line RSL65 that conferred partial resistance (IT 2–4) at high temperature were completely susceptible (IT 8–9) at the 10/15 °C cycle. A consistent IT score (IT 0–1) was obtained at the 10/15 °C cycle for the six accessions, which showed hypersensitive response (IT 1) or immune (IT 0) reaction at

Table 2 Frequency of each SNP in the *WKS1* gene of 55 *T. dicoccoides* accessions reported in this study plus the original sequence of RSL65

SNP no.	Frequency	Nucleotide substitutions	Amino acid substitutions	Note
SNP941	0.0182	G → A	Arg → His	Exon1
SNP1115	0.0182	A → –	nc	Intron1
SNP1194	0.3636	– → A	nc	Intron1
SNP1322	0.7455	T → A	nc	Intron1
SNP1349	0.0182	C → A	nc	Intron1
SNP1564	0.0364	T → A	nc	Intron1
SNP2169	0.0182	– → T	nc	Intron1
SNP2180	0.0545	C → –	nc	Intron1
SNP2320	0.4364	T → A	nc	Intron1
SNP2598	0.0364	T → C	Trp → Arg	Exon2
SNP3738	0.9818	G → A	Gly → Glu	Exon3
SNP7058	0.1091	T → C	nc	Intron10
SNP7257	0.0364	C → T	–[a]	Exon11

nc none-coding sequence

[a] No change in amino acid

Table 3 Nucleotide diversity and neutrality test of different regions along the *WKS1* gene sequence

Region[a]	Fragment length (bp)	π[b]	θ_w	Tajima' *D*	Fu and Li's *D*	Fu and Li's *F*	*K*
Coding region	1938	0.00011	0.00045	−1.67496	−1.24009	−1.60660	0.21500
Intron	5351	0.00022	0.00020	0.21847	0.11052	0.16919	1.19330
Exon1	996	0.00004	0.00022	−1.09306	−1.86875	−1.90315	0.03600
Intron1	1554	0.00064	0.00056	0.30779	−0.12246	0.01238	0.99500
Exon2	107	0.00067	0.00204	−0.88298	0.53512	0.14369	0.07100
Exon3	77	0.00047	0.00284	−1.09360	−1.86875	−1.90315	0.03640
Intron10	1548	0.00013	0.00014	−0.12346	0.53512	0.39852	0.19800
Exon11	125	0.00057	0.00175	−0.88298	0.53512	0.14369	0.07100
Entire region	7289	0.00019	0.00027	−0.78073	−0.67070	−0.83316	1.40875

[a] Coding region, all exons combined; *Intron* all introns combined, *Entire region* the full length genomic region of *WKS1*

[b] π nucleotide diversity (Nei and Li 1979); θ_w per-site estimates of diversity by Watterson's theta; *K* average nucleotide difference; The values of monomorphic introns 3, 4, 5, 6, 7, 8 and 9, and exons 4, 5, 6, 7, 8, 9, 10 of *WKS1* are not shown in this table

high temperature. The accession with the WKS1_P2 protein was susceptible (IT 9) to the stripe rust at low temperature as well (Supplementary file 5).

Discussion

The cloning of *Yr36* (*WKS1*) gene in wheat revealed a unique protein structure combining a kinase domain with a putative START lipid-binding domain that together confer resistance to a broad spectrum of wheat stripe rust races at relatively high temperatures (Fu et al. 2009). The *WKS2* paralog adjacent to *WKS1* with the 3.5 kb TE at intron 10 was identified as a non-functional gene (Gou et al. 2015). Furthermore, the two *WKS* genes were not detected in cultivated tetraploid or hexaploid wheat and, therefore, it is assumed they were not incorporated into cultivated wheat gene pool during the initial events of wheat domestication. In the present study, we studied the distribution of *WKS* genes in a large set of *T. dicoccoides* accessions and analyzed the nucleotide polymorphisms and haplotype diversity with respect to the functional *WKS1* allele.

Distribution of *WKS* genes in *T. dicoccoides* natural populations

The results of the current survey of 435 *T. dicoccoides* accessions revealed that a high percentage of the accessions from Israel (68 %), Syria (80 %), and Jordan (43 %) carry the *WKS* genes, while only 14 % of the accessions from Lebanon were positive, and none of the *T. dicoccoides* accessions from Turkey and Iran carry any of the two *WKS* genes. Our results are consistent with those described before by Fu et al. (2009). The previous study, including

Table 4 SNP-based haplotypes of the DNA sequence of *WKS1* gene found in natural populations of *T. dicoccoides*

Haplotypes	Nucleotide position[a]													CDS haplotypes	Protein types
	941	1115	1194	1322	1349	1564	2169	2180	2320	**2598**	**3738**	7058	**7257**		
Hap0	**G**	A	–	T	C	T	–	C	T	**T**	**G**	T	**C**	CDS_Hap0	P0
Hap1											A			CDS_Hap1	P1
Hap2							T				A			CDS_Hap1	P1
Hap3				A							A			CDS_Hap1	P1
Hap4				A						C	A			CDS_Hap3	P3
Hap5	A			A							A			CDS_Hap2	P2
Hap6				A		A					A			CDS_Hap1	P1
Hap7			A	A						C	A			CDS_Hap3	P3
Hap8				A					A		A			CDS_Hap1	P1
Hap9		–	A	A					A		A	C	T	CDS_Hap4	P1
Hap10			A	A					A		A		T	CDS_Hap4	P1
Hap11			A	A					A		A			CDS_Hap1	P1
Hap12			A	A	A				A		A			CDS_Hap1	P1
Hap13			A	A					A		A	C		CDS_Hap1	P1
Hap14			A	A				–	A		A			CDS_Hap1	P1
Hap15				A				–	A		A			CDS_Hap1	P1

[a] Nucleotide positions are relative to the start codon of the *WKS1* gene. The nucleotide positions located on exons of *WKS1* are indicated in bold. The original genomic sequence of *WKS1* from RSL65 was defined as Hap0

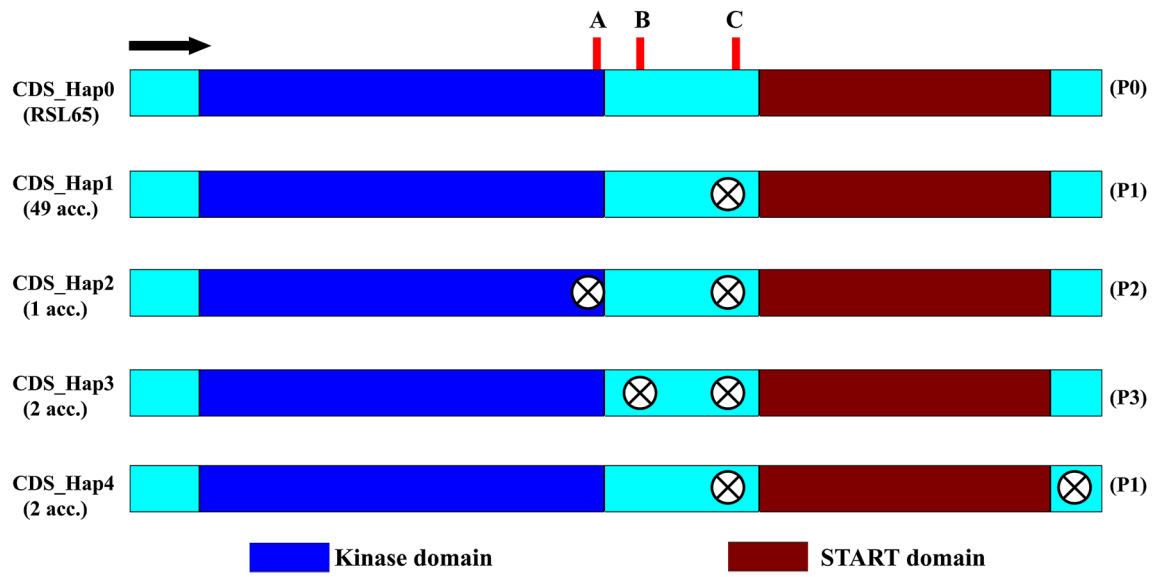

Fig. 2 Schematic representation of different coding sequence haplotypes of *WKS1* in *T. dicoccoides* accessions. From 54 cDNA sequences, four different coding sequence haplotypes (CDS_Hap1-4) were identified based on their sequence divergence. The coding sequence haplotype of the published *WKS1* is defined here as CDS_Hap0. The number of accessions corresponding to each haplotype is indicated in *brackets* on the *left*. SNPs in each CDS haplotype are indicated by *circled* X. Four different *WKS1* proteins corresponding to five CDS haplotypes are indicated on the *right* (P0, P1, P2, and P3). *Red bars* represent polymorphic amino acids changes compared with the functional *WKS1* protein from RSL65 published in the initial study (P0): *A* R314H; *B* W349R; *C* G373E

a more limited sample of 68 accessions of *T. dicoccoides* from the Fertile Crescent, detected the *WKS* genes only in accessions from Israel and southern Syria (southern distribution range of *T. dicoccoides*) but not in Turkey, Iraq, and Iran (northern distribution range of this species). Previous studies have shown that domesticated wheat is most closely related to the northern populations of *T. dicoccoides* (Luo et al. 2007; Ozkan et al. 2002, 2005). Therefore, the

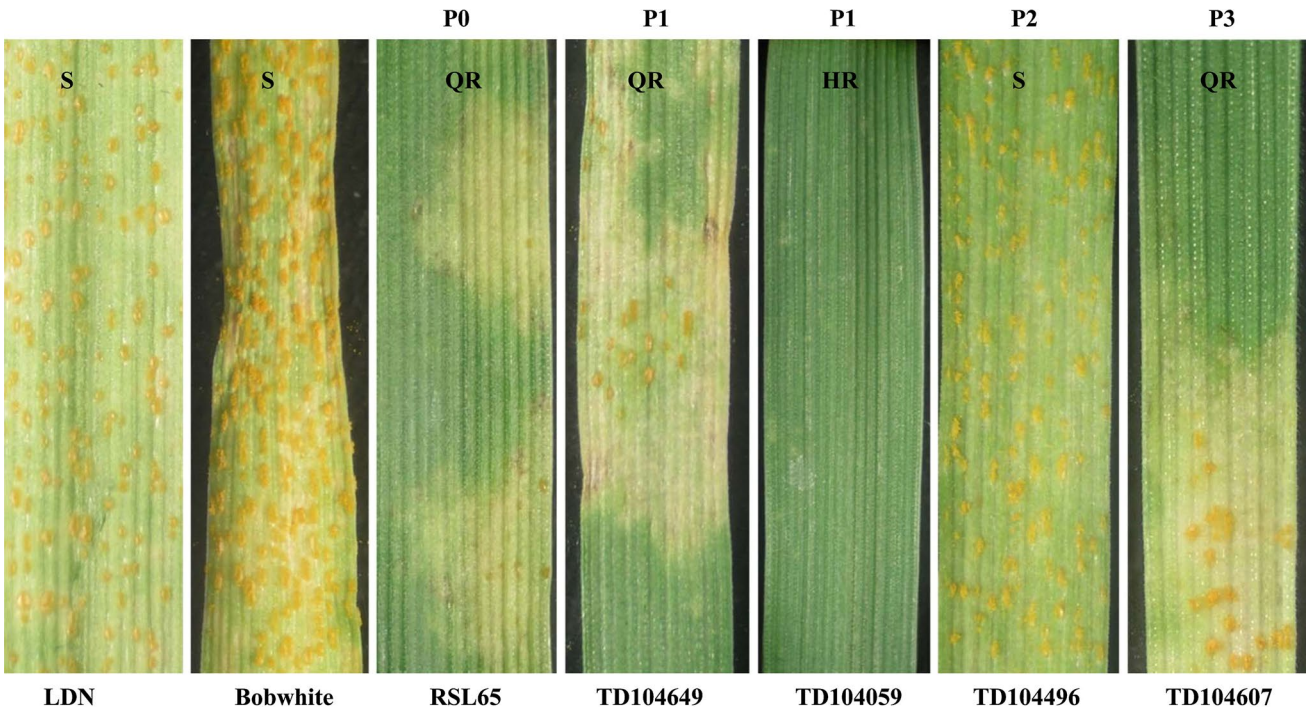

Fig. 3 Stripe rust resistance phenotype in *T. dicoccoides* accessions carrying different *WKS1* alleles. All of the plants were inoculated at the fourth leaf stage with *Pst* isolate 5006 (race 38E134) and were kept at a temperature regime of 10/25 °C night/day. The susceptible wheat lines LDN and Bobwhite were used as negative controls (absence of *WKS1*). RSL65 that carries functional WKS1 (P0) was used as a positive control. *T. dicoccoides* accessions TD104649, TD104059, TD104496, and TD104607 represent *WKS1* alleles P1, P1, P2, and P3, respectively. Three types of responses to inoculation with *Pst* were observed: *S* susceptible (IT 8–9), *QR* quantitative resistance (IT 2–4), *HR* hypersensitive response (IT 1)

absence of *WKS* genes in *T. dicoccoides* accessions that reside in the northern distribution range, as well as in all of the cultivated wheat gene pool, support the idea that wheat domestication was initiated in southeastern Turkey where the *WKS* genes are absent.

The *WKS* genes have been detected in other Triticeae species (Fu et al. 2009), indicating that the presence of these genes in *T. dicoccoides* represent the ancestral state. Therefore, the absence of *WKS* from the northern range of distribution of wild emmer in the Fertile Crescent indicates that these genes were lost in this region prior to domestication. Different hypotheses may explain this observation: (1) the presence of other *Pst* resistance genes in the northern distribution range that make *WKS1* redundant; (2) the climate in the northern regions is colder, and temperatures during the growing season are not high enough to allow the expression of the resistance of *WKS1*. Additional studies are required to test these hypotheses.

The screening of a large collection of *T. dicoccoides* accessions revealed that *WKS* genes are present in most of the collection sites in Israel and Syria across a diversity of environments. In the southern part of Israel, however, *WKS* genes are absent in most of the populations (8 out of 13), while present in most of the northern Israeli populations (104 out of 112) (Fig. 1). The correlation analysis of the climatic variables against the frequency of *WKS* genes shows that *WKS* frequency is higher in habitats with lower winter temperatures and higher precipitation conditions, which are the favorable conditions for development of stripe rust (Chen 2005). In Israel, the climatic conditions for stripe rust development are better in the northern region, which is cooler and more humid, during the wheat growing season, than the southern parts of Israel. Therefore, there is less selection pressure of the pathogen to maintain *WKS1* in the southern populations and it may have been lost during the evolutionary history of these wild emmer populations.

In the north part of Israel, however, the distribution of *WKS1* is low in the Mt. Hermon region. The climate of this region, with a high elevation (>1000 m), is very cold during the winter, and the maximum temperature in March does not exceed 13 °C. Therefore, *WKS1* may not be active in these regions and probably was not maintained in the Hermon population. Furthermore, the majority of wild emmer accessions collected from this region have qualitative resistance to *Pst* that eliminates the selective advantage of *WKS1* (Cheng et al. 2010). In the center of wild emmer distribution, the Golan Heights and Eastern Galilee, half of the populations are still polymorphic for the presence/absence

(P/A) of *WKS1*. In other sites, *WKS1* can be present in all tested accessions in one site, while completely absent from a neighboring site.

The fact that *WKS1* is absent in most Triticeae lineages suggests that there is likely a cost for the presence of this resistance gene. A recent study showed that overexpression of *WKS1* results in accelerated leaf senescence associated to the downregulation of the ability of the plant to detoxify reactive oxygen species (Gou et al. 2015). This increased susceptibility to oxidative stress may be associated with a negative selection for *WKS1* in the absence of the pathogen, or in the presence of more effective major genes that confer resistance to stripe rust (e.g. *Yr15*, Sun et al. 1997; *YrH52*, Peng et al. 1999). This negative cost of *WKS1* may result in balancing selection in the presence of the pathogen (Bergelson and Purrington 1996; Stahl et al. 1999; Shen et al. 2006).

We currently do not know if the fitness cost is associated to *WKS1* or to a linked gene. *WKS1* is closely linked (0.3 cM) to the *GPC-B1* gene that accelerates senescence and nutrient remobilization (Uauy et al. 2005). However, the presence of the chromosome region including both *Yr36* and *Gpc-B1* was associated with reductions in grain weight and yield in field trials in nine pairs of near isogenic wheat lines (Brevis and Dubcovsky 2010). The two genes have now been separated by recombination (Hale et al. 2013); therefore, now it will be possible to separate the effects of these two linked genes on grain weight and yield.

Reduced genetic diversity at the *WKS1* locus

In the current study, we detected a very low nucleotide diversity of *WKS1* in 54 *T. dicoccoides* accessions. *WKS1* alleles showed an average nucleotide diversity of $\pi = 0.00019$, which is about 14 times lower than the mean nucleotide diversity ($\pi = 0.0027$) in 21 *T. dicoccoides* genes (e.g. amino acid permease, grain softness protein, ATP and carotenoid biosynthesis relate genes; Haudry et al. 2007). A similar low level of polymorphism has been reported in some of the genes that control the activation of defense response in *Arabidopsis thaliana* and in some Scots pine populations at the *pal1* locus encoding phenylalanine ammonia-lyase (Bakker et al. 2008; Dvornyk et al. 2002).

In the current study, we have identified only 13 SNPs along the >7 kb full-length nucleotide sequence of *WKS1* within a collection of 54 accessions that represent a range of the natural habitats where *T. dicoccoides* is currently found. Furthermore, most of the variants were found in non-coding intron regions. A high level of sequence conservation was found also in *Lr34*, which is a putative ABC transporter that confers durable resistance to multiple fungal pathogens in wheat, including leaf rust (*Lr34*), stripe rust (*Yr18*), and powdery mildew (*Pm38*) (Krattinger et al. 2009). Alleles of resistant (*Lr34res-D*) and susceptible (*Lr34sus-D*) cultivars are characterized by only three mutations (Krattinger et al. 2009). Only five major *Lr34* haplotypes, either representing mutated versions of *Lr34res-D* or variants of *Lr34sus-D*, were identified in the wheat gene pool (Dakouri et al. 2010, 2014; Lagudah et al. 2009; McCallum et al. 2012). A recent study of a collection of 252 accessions of the D-genome progenitor of bread wheat, *Aegilops tauschii*, revealed very low nucleotide variation in *Lr34* ($\pi = 0.0011$; Krattinger et al. 2013), similar to the results presented here for *Yr36*.

The high level of conservation of *WKS1* found in *T. dicoccoides* natural populations is in sharp contrast to the high level of diversity found in race-specific R genes studied in *T. dicoccoides* populations, such as the NB-LRR genes. For example, the nucleotide diversities (π) detected in the leaf rust resistance gene *Lr10* ($\pi = 0.029$; Sela et al. 2011, 2012) and the powdery mildew resistance gene *Pm3* ($\pi = 0.007$; Sela et al. 2014) were 152 and 37 times higher than that of *Yr36* ($\pi = 0.00019$). The high levels of diversity found in NB-LRR resistance genes probably represent different specificities conferring resistance against various races of the pathogen. The race-specific R genes, such as *Lr10* and *Pm3*, are expected to have high diversity, due to continuous evolution of race-specific alleles to provide recognition of evolving pathogen effectors (Bergelson et al. 2001; Dangl and Jones 2001; Mauricio et al. 2003). Maintenance of allelic diversity at a specific R gene locus within natural plant populations occurs as a result of host-pathogen co-evolution (May and Anderson 1983). In contrast, partial resistance genes, such as *WKS1* and *Lr34* that provide resistance to a broad spectrum of pathogen races, are expected to be more conserved. It seems that there is no fitness advantage for new mutations and specificities to evolve, when a single allele is conferring resistance to most or even all of the pathogen isolates. The localization of WKS1 inside the chloroplast (Gou et al. 2015) suggests that this gene is monitoring a change in plant metabolism (e.g. a change in the signaling lipids sensed by the START domain) rather than a specific effector of the pathogen. The absence of diversifying selection operating on WKS1 may be associated to its low level of diversity compared with the NB-LRR R genes.

Potential functional *WKS1* allele in *T. dicoccoides* populations

In the present study, three different WKS1 protein sequences were predicted from natural allelic variants. The WKS1 protein (P1), with one amino acid change relative to the RSL65 reference sequence (G373E), was found in a large number of *T. dicoccoides* accessions. The large

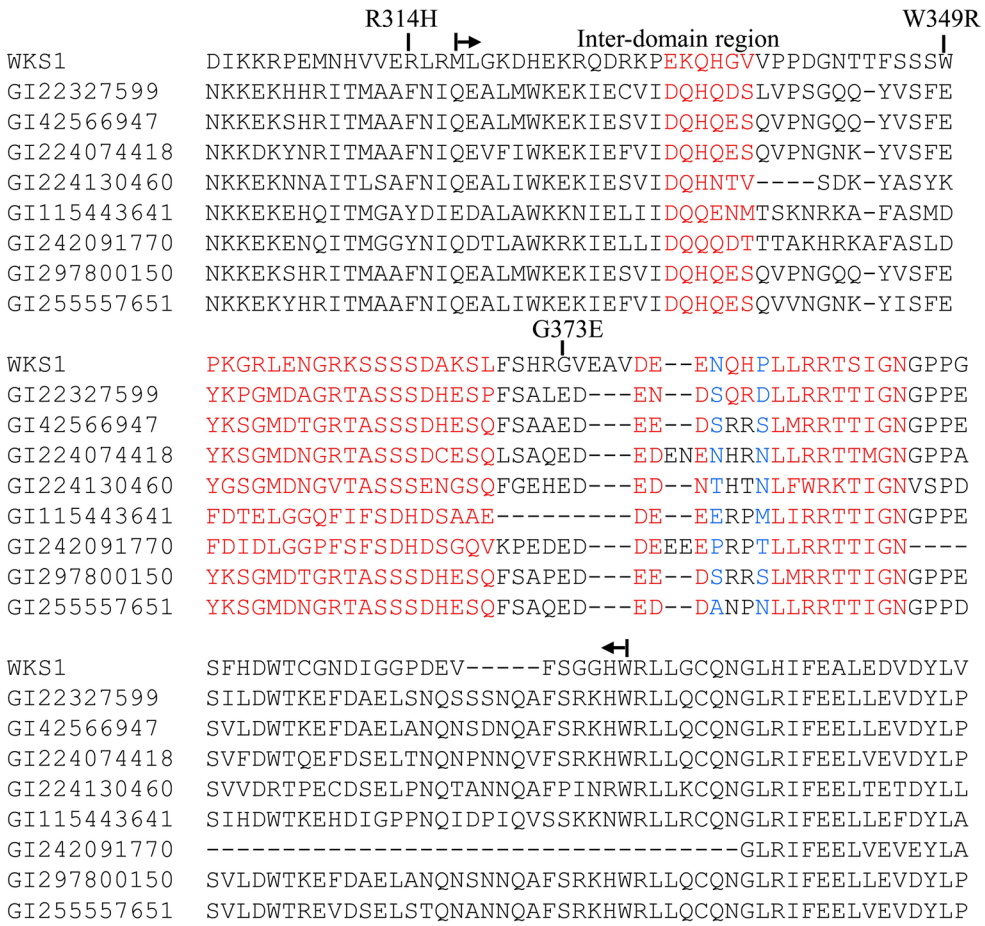

Fig. 4 Conservation patterns of the inter-domain region located between the kinase and the START domains of WKS1. *Arrows* indicate the inter-domain region of WKS1. WKS1 inter-domain region was aligned against the closest plant homologues from *Arabidopsis thaliana* (GI 22327599, GI 42566947, GI 297800150), rice (GI 115443641), *Populus trichocarpa* (GI 224074418, GI 224130460), *Sorghum bicolor* (GI 242091770), and *Ricinus communis* (GI 255557651). Most of the proteins aligned here to the WKS1 inter-domain region contained only a START conserved domain, without a kinase domain. Conserved motives detected within the WKS1 inter-domain region are marked in *red color*. The R314H, W349R, and G373E locations of WKS1 are also indicated. The site of glycine (G) at position 373 of WKS1 is commonly substituted by glutamic acid (E) in other proteins

number of accessions carrying the G373E polymorphism, together with the observation that the P2 and P3 haplotypes also carry the G373E polymorphisms indicates that (P1) is the ancestral haplotype, and that the original RSL65 WKS1 sequence (P0) represents a derived haplotype characterized by a G SNP at position 3738 (that results in the unique glycine amino acid at position 373). The G to E change in G373E is associated to a BLOSUM 62 score of 0 that indicates that the change is not very disruptive of structure or function. Sequence alignment of the inter-domain region between the kinase and the START domains of WKS1 with other similar defense proteins revealed that the mutation site (G373E) is not situated in a conserved region (Fig. 4). In addition, glutamic acid (E) at position 373 of WKS1 is more commonly observed at this position in other similar defense proteins than glycine (G) (Fig. 4). These findings suggest that the amino acid change (G373E) of P1, located in the space between the kinase, and the START conserved domains is not critical for the function of this protein.

Accessions with the P1 haplotype showed a partial resistance response at 10/25 °C, but susceptible reactions at 10/15 °C, similar to the results published for the WKS1 protein (P0) originated in RSL65 (Fu et al. 2009). These results suggest that the WKS1 protein (P1) is functional and confers resistance to stripe rust. However, resistance in some of these accessions may come from other genes, as we have observed for some accessions with P1 that have shown a hypersensitive response typical to NB-LRR R genes (Fig. 3; Supplementary file 5).

Protein (P3) shows an amino acid change of W349R located in the inter-domain region between the kinase and START conserved domains, in addition to G373E present

in P1. The W > R BLOSUM62 (Henikoff and Henikoff 1992) score is −3, which indicates a rare replacement that likely disrupts protein function. However, this change is in a non-conserved position, which may limit the impact of the mutation. The WKS1 protein P3 was found in two independent accessions originated from different collection sites in Israel. These two accessions showed partial resistance similar to the original *WKS1* (Fig. 3; Supplementary file 5) suggesting that the P3 protein is also functional.

Only one accession carried the WKS1 protein P2 that had two amino acid changes relative to the published WKS1 sequence (P0, R314H, and G373E). This accession showed a complete susceptible response, both under low and high temperature regimes (Fig. 3). Since the G373E polymorphism is also present in the P1 resistant haplotype, the susceptible response is most likely caused by the amino acid replacement (R314H) located in the kinase domain of *WKS1*. This loss of function was unexpected based on the bioinformatic analyses. The 314 position is not conserved among proteins having this domain (NCBI cd14066) and the R > H substitution has BLOSUM62 value of 0, which indicates a limited effect on protein structure. However, it seems that WKS1 is very sensitive to changes in amino acid in the kinase domain. In addition to the non-functional allele (P2) described here that was found in natural populations of wild emmer, Fu et al. (2009) found four independent amino acid changes in the kinase domain, artificially induced by EMS treatment, that disrupted the function of the protein, as evident from the resulted susceptible response of all four independent mutants.

Conclusions

The results presented here and in previous studies show the importance of wild emmer wheat as a valuable source for the improvement of stripe rust resistance in cultivated wheat (e.g. Cheng et al. 2010; Fahima et al. 1998; Fu et al. 2009; Nevo et al. 2002; Uauy et al. 2005). *WKS* genes were detected only in the southern distribution range of wild emmer natural populations in the Fertile Crescent (e.g. Israel, Syria, Jordan, and Lebanon). The low level of *WKS1* diversity among wild emmer natural populations compared with the NB-LRR R genes (e.g. *Lr10* and *Pm3*) probably reflects the different evolutionary forces acting on these genes due to the different mechanisms underlying the resistance response conferred by these genes. The different resistance response to stripe rust observed in the WKS1 proteins in all of the tested wild emmer accessions emphasizes that the WKS1 proteins (P0, P1, and P3) are functional in conferring resistance to stripe rust and can be further exploited for wheat improvement. These studies demonstrate the high potential of wild emmer wheat gene pool for improvement of durum and bread wheat resistance to this devastating pathogen.

Author contribution statement L. H., H. S., T. K., J. D., and T. F. designed the experiments. L. H., L. H. F., and Q. J. C. performed the experiments. L. H., H. S., L. H. F., T. K., J. Y., J. D., and T. F. discussed results and wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments comply with the current laws of the country in which they were carried out.

Accession numbers Sequences were deposited in the National Center for Biotechnology Information GenBank under accession numbers KT834954 to KT834968.

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