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ORIGINAL PAPER

A non-additive interaction in a single locus causes a very short root phenotype in wheat

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Abstract Non-additive allelic interactions underlie over dominant and under dominant inheritance, which explain positive and negative heterosis. These heteroses are often observed in the aboveground traits, but rarely reported in root. We identified a very short root (VSR) phenotype in the F_1 hybrid between the common wheat (*Triticum aestivum* L.) landrace Chinese Spring and synthetic wheat accession TA4152-71. When germinated in tap water, primary roots of the parental lines reached ~15 cm 10 days after germination, but those of the F_1 hybrid were ~3 cm long. Selfing populations segregated at a 1 (long-root) to 1 (short-root) ratio, indicating that VSR is controlled by a non-additive interaction between two alleles in a single gene locus, designated as *Vsr1*. Genome mapping localized the *Vsr1* locus in a 3.8-cM interval delimited by markers *XWL954* and *XWL2506* on chromosome arm 5DL. When planted in vermiculite with supplemental fertilizer, the F_1 hybrid had normal root growth, virtually identical to the parental lines, but the advanced backcrossing populations segregated for VSR, indicating that the F_1 VSR expression was suppressed by interactions between other genes in the parental background and the vermiculite conditions. Preliminary physiological analyses showed that the VSR suppression is independent of light status but related to potassium homeostasis. Phenotyping additional hybrids between common wheat and synthetics revealed a high VSR frequency and their segregation data suggested more *Vsr* loci involved. Because the VSR plants can be regularly maintained and readily phenotyped at the early developmental stage, it provides a model for studies of non-additive interactions in wheat.

G. S. Challa and Z. Zhang contributed equally to this research.

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Introduction

Non-additive interactions have long interested plant breeders and evolutionary biologists because they underlie hybrid vigor and hybrid incompatibility. While hybrid vigor provides the basis for hybrid breeding, hybrid incompatibility poses a mechanism of post-zygotic isolation to gene flow within and between species, the Bateson–Dubzhansky–Muller (BDM) model of speciation. Hybrid incompatibility includes hybrid lethality, hybrid necrosis, and hybrid sterility, and usually involves one or more genes from each parent. Since the first observation in tobacco (Brieger 1929), hybrid weakness, including hybrid necrosis, chlorosis, hybrid tumor formation and viral activation incompatibilities, have been described in 26 genera (reviewed by Bomblies and Weigel 2007). More than a century after the initial proposal by Bateson (1909), cloning of the BDM genes started to shed light on the genetic and molecular mechanisms of the hybrid incompatibility in plants. Similar to the BDM genes in Drosophila, plant BDM genes are fast-evolving (Bomblies and Weigel 2007).

In inter-specific crosses of tomato (Krüger et al. 2002) and intra-specific crosses of Arabidopsis (Bomblies et al. 2007), disease resistance (*R*) genes are involved in the hybrid necrosis interactions. *R* genes are usually organized into clusters and evolve rapidly, driven by dynamics of the pathogen populations (Michelmore and Meyers 1998). RIN4, the well-known guardian interacting with multiple R proteins, is involved in a necrotic interaction in an inter-specific cross of lettuce (Jeuken et al. 2009). All this supports a general notion that hybrid necrosis is an auto-immune syndrome as a byproduct of adaptive selection for disease resistance (Bomblies and Weigel 2007). Intimately related with the *R* gene families in function and structure, receptor-like kinase (*RLK*) genes constitute another class of BDM candidates. Interaction between the two alleles at an *RLK* gene locus, *OAK*, triggered programmed cell death (PCD) and outgrowth in intra-specific Arabidopsis hybrids (Smith et al. 2011). In addition, WRKY transcription factor TTG2 is involved in hybrid embryo lethal in Arabidopsis (Dilkes et al. 2008), an interaction between two alleles in an aspartic protease gene locus caused *indica–japonica* hybrid female sterility (Chen et al. 2008), and an interaction between two adjacent genes encoding an E3 ligase-like protein and an F-box protein led to *indica–japonica* hybrid male sterility in rice (Long et al. 2008). In another aspect, an overdominant interaction between the functional and nonfunctional alleles at the SFT locus of tomato increased fruit yield by ~60 % (Krieger et al. 2010). Genetically, heterozygosity fitness is not always consistent with gene activity as demonstrated in the *OAK* locus, where the aberrant development was due to increased affinity of the protein heterodimer to a natural substrate and ectopic activation of the downstream signaling pathway (Smith et al. 2011).

Common wheat or bread wheat (*Triticum aestivum* L., genomes AABBDD) is a hexaploid species originated from crosses between tetraploid wheat (*T. turgidum* L., genomes AABB) and diploid goatgrass (*Aegilops tauschii* Coss., genomes DD) (Kihara 1944; McFadden and Sears 1946). It is a classical model for polyploidy speciation and hybrid incompatibility studies. Several types of hybrid weakness have been recognized in wheat, including hybrid necrosis (Heyne et al. 1943; Hermsen 1963), hybrid chlorosis (Hermsen 1963), and grass-clump dwarfness (Canvin and McVetty 1976). As the best characterized type of hybrid weakness, hybrid necrosis in wheat is expressed as gradual premature death of leaves or the whole plant. It is caused by the interaction of two dominant genes, *Ne1* on chromosome arm 5BL and *Ne2* on 2BS (Tsunewaki 1970; Chu et al. 2006). The necrotic allele of *Ne2* is tightly linked with several rust resistance genes and its frequency in improved varieties has dramatically risen in the last 100 years (Pukhalskiy et al. 2000), suggesting a purifying selection on this locus. Abundant accumulation of reactive oxygen species (ROS) in plants carrying both *Ne1* and *Ne2* indicates that the PCD observed was mediated by a hypersensitive response (Sugie et al. 2007). Recently, two different types of hybrid necrosis were characterized in inter-specific crosses between durum wheat and goatgrass, which represent inhibitors to hexaploid wheat speciation (Mizuno et al. 2010, 2011). The goatgrass causal genes, *Nec1* and *Net2* are located on chromosome arms 7DS and 2DS, respectively. In the *Nec1*-mediated necrosis, reduction in photosynthesis was followed by ROS accumulation and PCD (Mizuno et al. 2010). Compared to *Nec1*, *Net2*-mediated necrosis is also involved in PCD but induced by low temperature (Mizuno et al. 2011). All this suggests that hybrid necrosis is controlled by a conserved mechanism, i.e., autoimmunity, in wheat.

To date, all the hybrid weakness cases were observed in the aerial organs except one case in rice, where hybrid weakness was found in both aboveground and underground tissues including reduced plant height, reduced root length but increased root number (Chu and Oka 1972). This situation is mainly caused by the fact that the aboveground tissues receive the most attention for intensive improvement of yield, quality and disease resistance and need much less effort and cost for phenotyping as compared to the root traits. We observed a very short root (VSR) phenotype in F_1 hybrid derived from a cross between the common wheat landrace Chinese Spring (CS) and synthetic wheat accession TA4152-71 (TA). Because it strongly contrasts the long roots of both parents, VSR represents a non-additive interaction between the parental genomes in the F_1 hybrid. We characterized VSR by genetic analysis, genome mapping and physiological approaches. Here we report the results and implications in root development and plant nutrition.

Materials and methods

Plants and growth conditions

All the plant materials used are listed in Supplementary Table 1s. Wheat plants were grown in 4″ × 4″ square pots containing Sunshine® potting mix #3 (Sun Gro Horticulture) supplemented with Multicote®8 Controlled-Release Fertilizer (Haifa) in a greenhouse. The temperature in the greenhouse was 22 °C during the day and 17 °C at night and the day length was 16 h. Crosses were made by manual emasculation and pollination.

For root phenotyping, seeds were germinated in 4″ × 4″ germination boxes (Durphy Packaging Co.) containing 10 ml tap water at room temperature in the laboratory, or directly planted in deep pots containing vermiculite and perlite (Hummert) mixed at a 2:1 ratio and supplemented

with a layer of the Multicote®8 Controlled-Release Fertilizer. Root phenotypes in germination boxes were determined 7 days after germination (DAG), and phenotyping of plants growing in the deep pots was conducted 20 days after planting by carefully removing them with vermiculite, rinsing in water and measuring the root length. After root phenotypes scored, the plants were transplanted in the 4″ × 4″ square pots. For testing mineral nutrient effect on VSR, CS, TA and their F_1 hybrid were germinated in 1 mM KCl. For testing light effect on VSR, the germination boxes were covered with foil and placed in a drawer. The genotype of the F_1 hybrid was confirmed by red coleoptiles, a trait inherited from the male parent TA.

Marker development, genotyping and mapping

Rice genes from *Os03g63110* through *Os03g63510* were retrieved from Rice Genome Annotation database (http://rice.plantbiology.msu.edu/) and used as queries to search the wheat Gene Index database (http://compbio.dfci.harvard.edu/tgi/tgipage.html) and the wheat D-genome database WheatDB (http://wheatdb.ucdavis.edu/). The wheat expressed sequence tags (ESTs) and D-genome sequence contigs were retrieved with minimal E-value of e-20 and minimal matched length of 300 bp. The ESTs and the genomic sequences showing highest homology were selected for primer designing using the online program Primer3 (http://frodo.wi.mit.edu/).

Leaves were collected for DNA isolation following Li et al. (2008). For marker genotyping, PCR was set up in 10 µl containing 1× reaction buffer (Promega), 1 µl diluted genomic DNA, 200 nM of each primer, 200 µM dNTPs and 0.1 Unit of *Taq* polymerase (Bioline). Following initial denaturation at 94 °C for 3 min, the reaction mix went through 35 cycles of denaturation at 94 °C for 30 s, annealing at 52–60 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR primers for the simple sequence repeat (SSR), cleaved amplified polymorphic sequence (CAPS) and sequence-tagged site (STS) markers are listed in Supplementary Table 2s. SSR and STS PCR products were separated by 6 % polyacrylamide gel electrophoresis. PCR products of CAPS markers TAG621 and WL938 were digested by *Taq*I before electrophoresis separation. Linkage maps were constructed using Mapmaker 3.0 (Lander et al. 1987) with the minimal LOD set at 2.0. The Kosambi (1944) function was used to transform recombination frequencies into centiMorgan (cM) distances.

Microscopy

Root tips were harvested from the seedlings 4 DAG, stained in 15 µM propidium iodide for 30 s and rinsed briefly in distilled water. The root tips were observed under a Fluoview FV300 Laser Scanning Confocal Microscope (Olympus).

Data analysis and statistics

The longest root was measured from each seedling. The means, standard deviations and *P* values were estimated using Microsoft® Excel functions. Student's *t* tests were performed to evaluate statistical significance of the differences between parental and F_1 plants, between control and treatment, or between different treatments. Pearson's Chi squared test was used to calculate the deviation of F_2 and BC_1F_1 segregations from the expected ratios. The cut-off for statistical significance was $P \leq 0.05$.

Results

Phenotypic characterization of VSR

Germinated in tap water, the F_1 hybrid derived from a cross between CS and synthetic wheat accession TA surprisingly showed a VSR phenotype. Subsequently, we germinated the remaining 46 F_1 seeds along with its parental lines and confirmed the contrasting difference (Fig. 1a). Compared to the white and sharp root tips of the parental lines, the root tips of the F_1 hybrid were blunt, fuzzy, even swollen and yellow- or brown-colored. CS and TA had the same growth rate ($P > 0.76947$), but the F_1 root growth rate was significantly reduced 2 DAG ($P < 4.6 \times 10^{-6}$; Fig. 1b). Lateral roots started to appear on VSR as early as 5 DAG, but no lateral roots appeared on the roots of the parental lines until 10 DAG. As a result, primary roots of CS, TA and their F_1 hybrid reached an average length of 15.1, 15.3 and 3.3 cm 10 DAG, respectively (Fig. 1a, b). Microscopic observation showed that the elongation zone in VSR of the F_1 was disrupted (Fig. 1c). Eventually all the roots in the F_1 hybrid lost their root apical meristem, which led to a very small root system architecture. These results indicate that root growth and development in the F_1 hybrid is arrested due to a non-additive interaction of dominant genes derived from the two parental genomes in the elongation zone and/or meristem. Although the F_1 hybrid showed shoot growth retardation at a very early stage in germination boxes (Fig. 1a), it grew regularly to maturity, set plenty seeds, and did not show significant symptom of hybrid weakness in the greenhouse condition probably because the F_1 root development was recovered in soil as discussed below.

Genetic analysis of VSR

Segregation ratios are routinely used to infer the genetic modes of the interacting alleles. A 9:7 ratio in an F_2

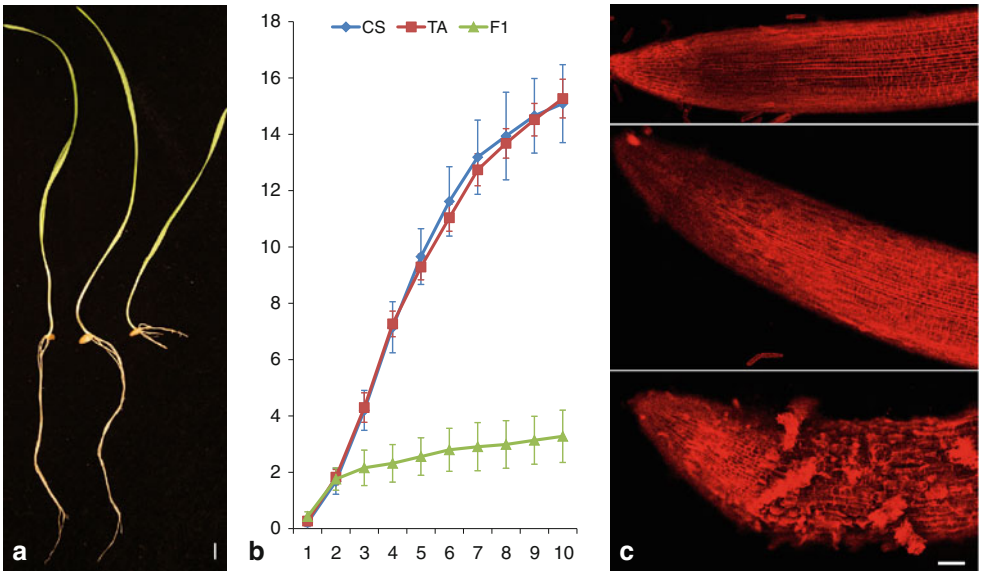

Fig. 1 Root phenotypes of CS, TA and their F_1 hybrid. **a** Wheat seedlings 7 days after germination, from *left* to *right*: CS, TA and F_1. The *bar* indicates 1 cm. **b** Root growth curves of CS, TA and F_1. The *numbers* in the *x*-axis indicate days after germination, the *numbers* in the *y*-axis indicate root length in cm, and the *error bars* indicate standard deviation of the means calculated from 10 or more biological replicates. **c** Confocal images of root tips that were stained in 15 μM propidium iodide. From *top* to *bottom*: CS, TA and the F_1. The *scale bar* indicates 50 μm

population predicts a complementation between two unlinked dominant genes and a 1:1 ratio predicts an interaction between two closely linked dominant genes or two codominant alleles at the same locus. When germinated in germination boxes, two types of roots were found in the F_2 population, long root (parental type) and short root (hybrid type), and no intermediate type was observed. Of the 583 F_2 seedlings, 293 had long roots and 290 had short roots, which fits a 1:1 ratio ($P > 0.92034$) and significantly differs from a 9:7 ratio ($P < 0.00396$). This strongly suggests that two co-dominant alleles at one locus or the dominant alleles of two tightly linked genes from the two parents are involved in the F_1 phenotype. For convenience of discussion, we hypothesize that a non-additive interaction between two alleles in a single locus underlies the VSR phenotype. According to the recommended rules for gene symbolization in wheat (McIntosh et al. 2008), we designated locus as *Vsr1*, the CS allele as *Vsr1a* and the TA allele as *Vsr1b*.

The synthetic wheat accession TA is the amphiploid derived from a cross between durum wheat cultivar Trinakria (PI 520121) and *Ae.tauschii* accession WX700. CS is a landrace, but the extracted tetraploid form (4×CS; 2n = 4× = 28, genomes AABB), which carries the A and B genomes nearly identical to those of CS, was developed by repeated backcrossing of CS to tetraploid wheat (Yang et al. 1999). To determine which genome harbors the *Vsr1* gene, we crossed CS with Trinakria and 4×CS with Trinakria. Compared to the VSR phenotype of the hexaploid F_1 hybrid, these pentaploid and tetraploid F_1 hybrids lacked at least one copy of the D genome and produced long roots (Fig. 2). This indicated that the *Vsr1* locus is located on a D-genome chromosome.

At the same time, we attempted to locate the *Vsr1* gene to a specific chromosome by crossing TA to the CS nullitetrasomics (NT) lines, in which loss of a chromosome pair is compensated by a double dose of its homoeologous chromosome (Sears 1966). We pollinated NT lines of the 21 chromosome pairs with TA, and all the F_1 hybrids showed VSR, suggesting that the absence of *Vsr1a* was compensated by the increased dosage of its homoeologs, or that one or more NT lines used were problematic. In the latter regard, N5D-T5A was one of the suspects based on its phenotype. The bona fide N5D-T5A has compact spikes due to the increased dosage of the *Q* gene on the chromosome 5A (Sears 1966). The N5D-T5A used in this experiment had a spike similar to that of euploid CS. This suggested a possibility that the N5D-T5A used was misidentified during the seed increase process, and chromosome 5D carried the *Vsr1* gene.

A total of 70 D-genome SSR markers, 10 markers on each of the seven D-genome chromosomes, were selected for screening polymorphisms between CS and TA. The genotyping data showed that 53 markers were polymorphic, indicating a polymorphism frequency of 77 %. Although expected, this is much higher than the D-genome variation among common wheat cultivars. We genotyped 94 F_2 VSR individuals using the polymorphic markers with

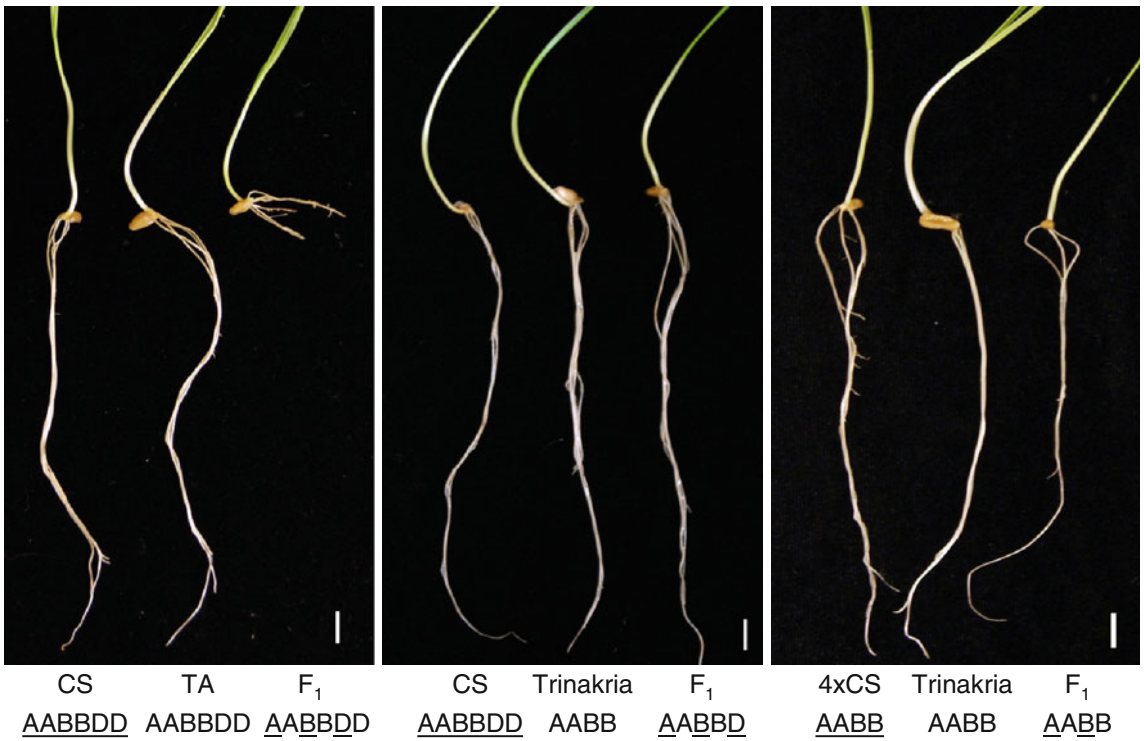

Fig. 2 Parental genome analysis of the *Vsr1* locus. Trinakria is the durum parent of synthetic wheat accession TA4152-71 (TA); and 4×CS is the extracted tetraploid of Chinese Spring (CS). Root growth was phenotyped 7 days after germination. Designation of parental lines and their F_1 hybrids, and their genome formula are indicated beneath the seedlings. The genomes derived from CS are *underlined*. The *scale bars* indicate 1 cm

an emphasis on chromosome 5D and detected close linkage between the *Vsr1* locus and the SSR marker *XBARC144*, which is known to be located in the distal region of chromosome arm 5DL (Somers et al. 2004; Song et al. 2005). Subsequently, SSR and STS markers from the same region were selected to increase map density. These included SSR markers *XBARC177*, *XGPW323*, *XWMC443* and *XWMC765*, and the STS marker *XTAG621*. The *Vsr1* locus was localized to an 8.8-cM interval, 1.1 cM above *XBARC144* and 7.7 cM below *XWMC765* (Fig. 3a).

Synteny-based marker development

To further increase marker density at the *Vsr1* locus, we developed additional markers based on the macrocolinearity between wheat and rice. On the International Triticeae Mapping Initiative (ITMI) map of chromosome 5D (http://wheat.pw.usda.gov/GG2/index.shtml), *XGPW323* mapped just below the restriction fragment length polymorphism (RFLP) marker locus *XBCD197*. A search of the rice genome (http://rice.plantbiology.msu.edu/) using the BCD197 clone sequence (GenBank accession BE438957) as a query found that it is homologous to the rice gene *Os03g63950*. We subsequently selected 19 rice genes above it, from the interval *Os03g63110* through *Os03g63510*, and retrieved wheat ESTs showing the highest homology to these rice genes for designing PCR primers. Marker WL954, developed from a wheat EST homologous to *Os03g63470*, detected polymorphism between CS and TA. Digestion of the PCR products of the remaining 18 primer pairs with 4-base cutters revealed that the WL938/*Taq*I combination also detected polymorphism. These two markers were used to genotype 241 VSR plants, including the 94 F_2 and 147 BC_1F_2 individuals, and mapped to the *Vsr1* interval, further reducing it to 7.1 cM (Fig. 3b). Analysis of the retrieved D-genome sequences found that sequence contigs homologous to rice genes *Os03g63140* and *Os03g63430* contained SSRs, which were developed into markers *WL2506* and *WL2510*, respectively. The former detected polymorphism between CS and TA, mapped between *XWL938* and *Vsr1*, and further narrowed the interval to 3.8 cM (Fig. 3b). Genotyping of 43 TA-backcrossed BC_1F_2 individuals using flanking markers BARC144 and WL2506 also confirmed this *Vsr1* location.

Alignment of wheat markers with their rice counterparts revealed an inversion involving *Os03g63140* and *Os03g63300* with one breakpoint located between *Os03g63300* and *Os03g63470* and another above *Os03g63140* (Fig. 3c). A search of the model grass *Brachypodium distachyon* genome at phytozome (www.phytozome.net) found that these genes are

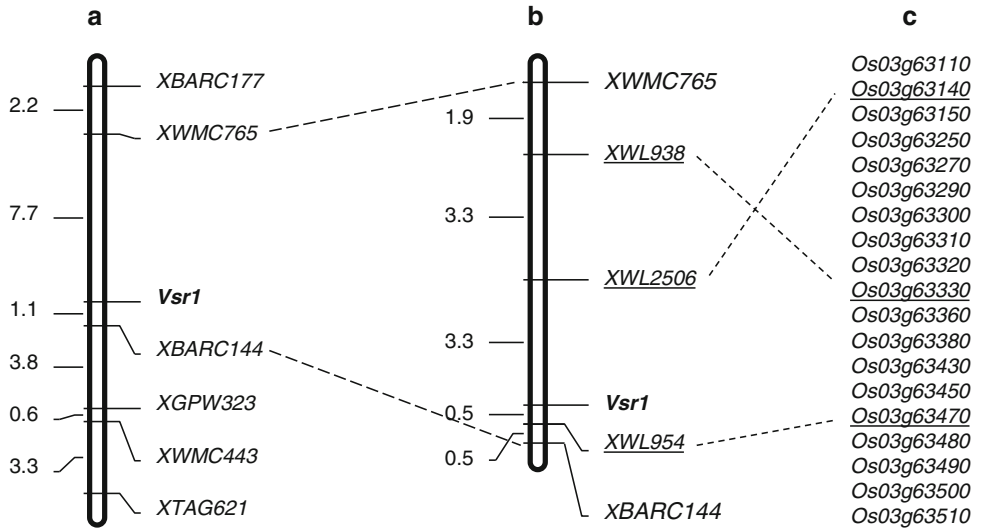

Fig. 3 Genetic maps of the *Vsr1* region on chromosome arm 5DL and colinearity with rice chromosome 3. **a** A map was constructed based on segregation of 94 VSR F_2 individuals; **b** a map based on the 94 F_2 and 147 BC_1F_2 VSR plants with newly developed SSR and STS markers. *Top* of the maps is towards the centromere and *bottom* towards the telomere. The numbers in the *left* of the maps are genetic distances in cM between the marker loci, which are listed in the *right* of the maps. The *Vsr1* locus is in *bold*. **c** Rice gene loci are listed in the same order as in the rice genome. The *dashed lines* between maps **a** and **b** link the same loci; and *dashed lines* between maps **b** and **c** link rice genes and their wheat homoeologous loci (*underlined*)

located in different chromosomes: homologs of *Os03g63140* and *Os03g63470* on chromosome 1, but homolog of *Os03g 63300* on chromosome 4. This suggests the *Vsr* region experienced numerous chromosome rearrangements during grass evolution.

VSR suppression

The VSR phenotype was observed when germinating seeds in germination boxes under laboratory conditions. We asked if it is also expressed in underground conditions. We tested this possibility by directly seeding the F_1 hybrid and the parental lines in deep pots containing vermiculite/perlite and a layer of fertilizer on the top. Phenotyping 20 days after planting turned out that their roots grew virtually identical and no VSR was observed in the F_1 hybrid (Fig. 4a). The genetic identity of the F_1 plants was confirmed by a combination of parental trait phenotypes, such as the red coleoptiles of TA and the awnless spike morphology of CS, as well as marker genotyping. However, VSR was observed in F_2 and backcross populations under the same conditions. In a population of 120 F_2 individuals, ~15 % plants showed short or VSRs and ~20 % were intermediate (Fig. 5). In the advanced backcross populations using either CS or TA as the recurrent parent, however, the proportion of long-root and short-root plants was roughly equal and no intermediate type was recovered (Fig. 4b). Marker genotyping of 96 CS-backcrossed BC_2F_1 individuals, 48 of long roots and 48 of short roots, indicated that an overwhelming majority of long-root plants

Fig. 4 Root phenotypes of seedlings 20 days after planting in vermiculite. **a** Parents and F_1 hybrid, from *left* to *right*: Trinakria, TA, CS and F_1 between CS and TA. **b** CS-backcrossed BC_1F_2 segregants, **c** TA-backcrossed BC_1F_2 segregants. The *scale bars* indicate 1 cm

had the CS allele at *XBARC144* locus and the short-root individuals were heterozygous (Fig. 6). This confirmed the effect of the *Vsr1* locus. Seeds from the short-root plants

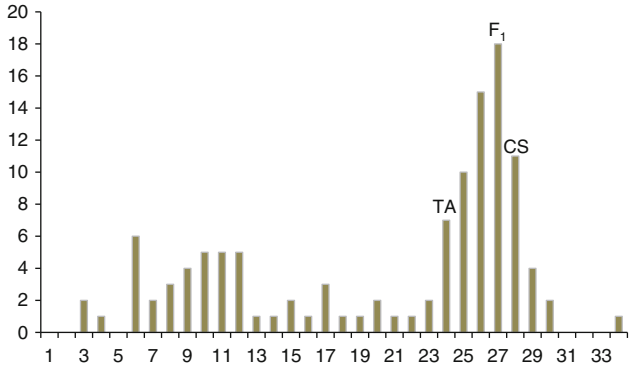

Fig. 5 Distribution of root length of an F_2 population derived from the cross between CS and TA 20 days after planting in vermiculite. The population consists of 120 F_2 individuals. The *numbers* in the *x*-axis indicate root length in cm, and the *numbers* in the *y*-axis indicate the numbers of the F_2 individuals with the same root length. Root lengths of CS, TA and the F_1 were measured at the same time and are indicated above the columns

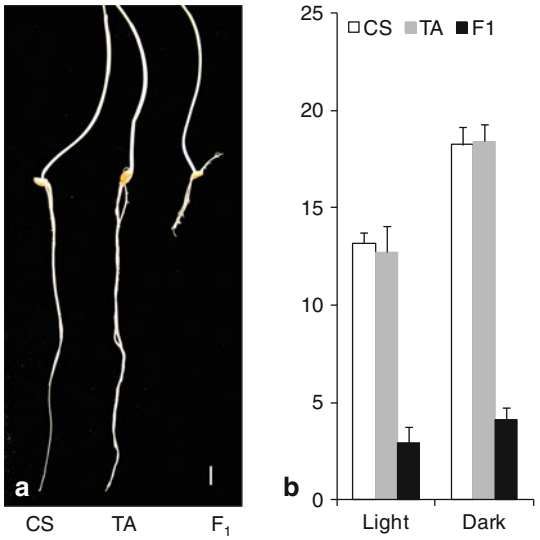

Fig. 7 Light effect on VSR development. **a** Roots of 7-day-old etiolated seedlings germinated and grown in darkness. The *scale bar* indicates 1 cm. **b** Comparison of root growth under light and in the dark. The *numbers* in the *y*-axis indicate root length in cm measured 7 DAG. The *error bars* indicate standard deviation of the means calculated from 10 or more biological replicates

continued to segregate for VSR. Collectively, these results indicate that the VSR expression in the F_1 plants was suppressed by other genes from either parent background when planted in deep pots.

Environment effects on VSR

We further asked what environmental factors contributed to the F_1 VSR suppression under the deep pot condition. One obvious difference between the germination box and deep pot conditions is the light status, which is involved in regulation of root development and growth (Sassi et al. 2012). To test the effect of light, we germinated seeds of the parents and F_1 in germination boxes, wrapped them with foil and placed in a drawer for a week. In the dark, the root phenotypic difference between the parents and the F_1 hybrid remained (Fig. 7a). Compared to the light condition, root growth of both parents and the F_1 hybrid increased in the dark ($P < 0.0028$), but darkness did not change the ratios of F_1 hybrid to parents (0.225) and even reduced the *P* values by more than six orders of magnitude, $P < 2.44 \times 10^{-12}$ in the light experiment and $P < 1.07 \times 10^{-18}$ in the dark experiment (Fig. 7b). This excluded the effect of light as being responsible for the VSR expression.

Another difference between the growing conditions lies in the availability and use of mineral nutrients because fertilizer was applied in the deep pots but not the germination boxes. Potassium (K^+) is an essential micronutrient and major component in the fertilizer applied and important for cell elongation (Christian et al. 2006), but it was at very low level in the tap water (Supplementary Table 3). In two separate experiments, we germinated CS, TA and the F_1 in deionized water as a control and in 1 mM KCl as a treatment. Considering single-salt toxicity, we only investigated K^+ effect on the early root growth, i.e., first 5 DAG. In both experiments, the F_1 root growth rate was significantly slower than those of the parental lines when germinated in deionized water ($P \leq 4 \times 10^{-5}$; Fig. 8). When germinated in 1 mM KCl, F_1 roots grew slower than the parental lines in experiment 1($P < 0.03165$) but similar to them in experiment 2 ($P > 0.17983$). Compared to germination in deionized water, root growth rate of the parental lines in KCl did not change in experiment 1 ($P > 0.05578$) but reduced in experiment 2 ($P < 0.02711$), but the F_1 root growth in KCl

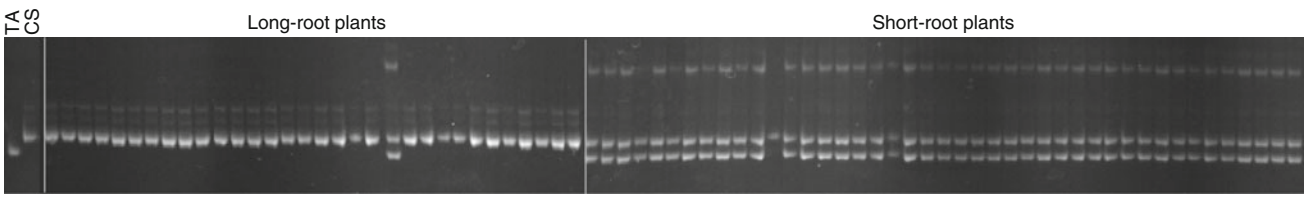

Fig. 6 BARC144 marker genotyping of parents and a portion of a CS-backcrossed BC_2F_1 population directly planted in deep pots. Parental lines and root phenotypes are indicated on the *top*. Most of the long-root plants had CS allele and most of the short-root plants were heterozygous

doubled in both experiments ($P < 0.00069$; Fig. 8). This indicates a possible role of K^+ nutrition in suppression of the F_1 VSR in the deep pot condition.

Occurrence of *Vsr* genes

The VSR phenotype was found in the F_1 between CS and TA accidentally. To determine if the *Vsr* genes exist in other common wheat cultivars and synthetic wheat accessions, we made 18 additional crosses between nine common wheat cultivars and 10 synthetic accessions. Of the 18 F_1 hybrids, eight showed VSR and the remaining 10 had normal root growth (Table 1), suggesting that the interacting *Vsr* alleles are widely distributed in populations of common wheat and *Ae. tauschii*. We phenotyped root growth of four F_2 populations and two small BC_1F_1 populations in germination boxes (Table 1). The two F_2 populations derived from the long-root F_1 hybrids did not segregate for VSR. Those F_2 and BC_1F_1 populations derived from the VSR F_1s segregated for VSR at ratios that differ from the 1:1 but fit the 3:1 expectation (Table 1; Supplementary Fig. 1s). These data suggested that additional *Vsr* loci may be involved in the interactions underlying the VSR phenotype in these four F_1 hybrids.

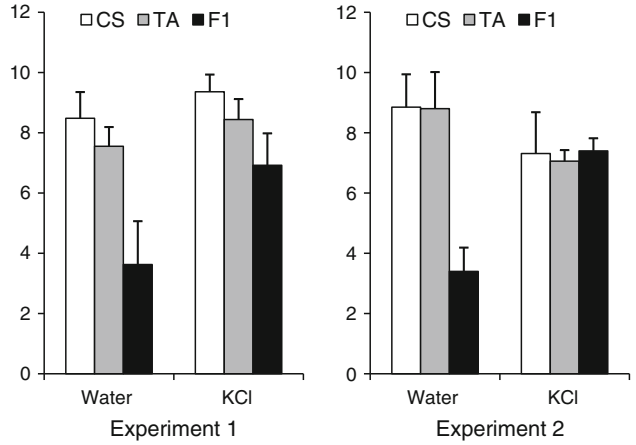

Fig. 8 Potassium effect on VSR development. Root growth in deionized water and in 1 mM KCl was compared in two separate experiments. The *numbers* in the *y*-axis indicate root length in cm measured 5 DAG. The *error bars* indicate standard deviation of the means calculated from 5 or more biological replicates

We also made four crosses between eight synthetic accessions, RWG18 × SW29, SW19 × SW44, SW53 × SW11, and TA3418 × TA3419. The first three F_1 hybrids produced long roots, and the F_1 hybrid between TA3419 and TA3436 had the VSR phenotype. These two synthetic wheat accessions had a common tetraploid parent (Tetra Canthatch) and therefore carry identical A and B genomes, but their D genomes were derived from two goatgrass accessions. A population of 98 F_2 individuals from this cross segregated into 54 long-root and 44 short-root plants, which fits a 1 to 1 ratio ($P = 0.36227$). This indicated that

Table 1 Root phenotypes of the F_1 hybrids between common wheat and synthetic wheat accessions and segregations in F_1, BC_1F_1 and F_2 generations

Common wheat parents	Synthetic wheat parents	F_1 seedlings	F_1 segregation (long:short)	F_2 segregation (long:short)	*P* values (1:1)
Brick	TA4152-71	6	6:0	105:0	0
Bobwhite	TA4152-71	7	0:7		
Bobwhite	TA4152-3	7	7:0		
Bobwhite	PI 648555	2	2:0		
Canthatch	TA4315	6	6:0		
Canthatch	TA4318	6	0:6	27:7*	0.00112
Canthatch	TA4336	6	0:6	19:8*	0.05441
Chinese Spring	TA4152-52	6	6:0		
Chinese Spring	TA4152-71	51	0:51	293:290	0.92034
Chinese Spring	SW44	6	6:0		
Chinese Spring	RWG18	7	7:0		
Chinese Spring	PI 648555	9	9:0		
Chinese Spring	PI 648480	4	0:4		
Komar	TA4152-71	3	0:3		
Louis	TA4152-71	4	0:4	80:19	0
Opata 85	TA4152-3	10	10:0		
Opata 85	TA4152-71	8	0:8	74:36	0.00042
Ramona 50	TA4152-71	3	0:3		
TA2609	SW44	8	8:0	100:0	0

* BC_1F_1 populations

differentiation at the *Vsr* loci occurred in the *Ae. tauschii* populations.

Discussion

Root systems provide plants water, nutrients, growth regulators, and soil anchorage. As the "hidden half" of a plant, however, root genetics has been hindered largely by the difficulties of invasive root phenotyping. Thanks to the rich genomic resources, simple root anatomy and nonsoil-medium cultivation, much progress of root biology has been made in the model plant Arabidopsis (Petricka et al. 2012). But we know very little about the genetic mechanisms of root development and growth in crops like wheat. In the present research, we identified a VSR phenotype in an F_1 hybrid and segregating populations of wide crosses between common wheat cultivars and synthetic wheat accessions. It contrasts parental root phenotype in slow growth rate, tip disruption and early emergence of lateral roots. Genetic analysis showed that VSR was resulted from an interaction between two alleles at a single locus *Vsr1*. This is the first observation of the VSR phenotype in wheat and a non-additive genetic interaction in a single locus regulating root development and growth in any plant. Genetic and physiological analyses provide insights into the mode of genetic interaction and environmental effects on root development.

Genetic mechanism of VSR

In the non-additive interactions, the two interacting alleles can either be located at a single locus (Chen et al. 2008; Krieger et al. 2010; Smith et al. 2011), at two linked (Long et al. 2008) or unlinked loci (Krüger et al. 2002; Bomblies et al. 2007; Jeuken et al. 2009). In wheat, hybrid weakness is caused by non-additive interactions between unlinked loci (Tsunewaki 1970; Chu et al. 2006; Mizuno et al. 2010, 2011). In the present research, ~1,000 VSR plants assayed were all heterozygous, supporting the single-locus hypothesis. However, there is also a possibility of pseudo-underdominance, i.e., two tightly linked loci in repulsion phase, as demonstrated by Long et al. (2008) in the *indica–japonica* hybrid male sterility locus, *Sa*, of rice. Cloning of the *Vsr1* locus will shed more lights on this.

Similar to segregation in the populations derived from the cross between CS and TA, a 1:1 segregation was also observed in an F_2 population derived from a cross between synthetic wheat accessions TA3418 and TA3419. In two F_2 and two BC_1F_1 populations derived from crosses between common wheat and synthetic wheat, however, the observed segregations are different from the 1:1 ratio and fit a 3:1 ratio, suggesting a possibility that four alleles in two unlinked loci are involved in the VSR interaction. Another factor contributing to deviation of the F_2 and BC_1F_1 segregations from the 1:1 ratio would be segregation distortion, which is frequently observed in the goatgrass (Faris et al. 1998) and wheat (Peng et al. 2000; Kumar et al. 2007). One type of segregation distortions is caused by female gametes preferentially fertilizing the male gametes carrying the same allele, which increases frequency of homozygous and reduces the heterozygous plants (Kumar et al. 2007). To clarify these two possibilities, we will need to genotype the VSR-derived F_3, BC_1F_2 and BC_2F_1 populations using the Vsr1-flanking markers.

As seen in Arabidopsis (Bomblies et al. 2007; Smith et al. 2011) and rice (Long et al. 2008), non-additive genetic interactions often occur in fast-evolving genomic regions. Alignment of the gene-based marker loci flanking *Vsr1* and their homologs in the model grasses Brachypodium and rice revealed colinearity breakdown. Although the orthologs are located in the long arm of rice chromosome 3, colinearity of these genes was disrupted by an inversion. In the Brachypodium genome, the orthologs are located in two different chromosomes. This suggested that the *Vsr1* region is highly divergent among the grasses. Comparative mapping of the *Vsr1* locus at a finer scale will provide more insights into the evolutionary history of this genomic region.

Autoimmunity is a prevailing feature of underdominance, including hybrid necrosis and outgrowth (Bomblies and Weigel 2007), in which *R* genes or related *RLK* genes are involved (Krüger et al. 2002; Bomblies et al. 2007; Jeuken et al. 2009; Smith et al. 2011). In wheat, *Ne1-Ne2, Nec1*- and *Net2*-mediated interactions caused ROS accumulation and PCD (Sugie et al. 2007; Mizuno et al. 2010, 2011). *Ne2* is associated with rust resistance and favored in the improved wheat cultivars (Pukhalskiy et al. 2000). Several disease resistance genes are located on 5DL, including *Lr1*, *Pm34*, and *Pm35*, of which *Pm34* falls in the *Vsr1* interval and between the marker loci *XBARC144* and *XBARC177* (Miranda et al. 2006). We crossed the *Pm34*-carrying wheat germplasm NC97BGTD7 (PI 604033) with CS, but the F_1 hybrid did not show VSR. Therefore, our current data did not provide evidence regarding whether VSR is related with biotic defense response, and more detailed research needs to be conducted for clarifying this.

Environmental effects on VSR

The VSR phenotype was discovered when germinating seeds in germination boxes with tap water. When directly planted in deep pots containing vermiculite with supplemental fertilizer, the F_1 hybrid between CS and TA did not show VSR, but its offspring segregated for it. Because tap water was also used to irrigate the plants in deep pots, VSR

suppression was probably resulted from induced expression of the suppressors in the parental background by the vermiculite/fertilizer condition. Two major factors were considered: light and mineral nutrition. In the dark, roots of parents and F_1 hybrid grew faster, confirming role of light in regulating root growth (Sassi et al. 2012). But the darkness did not change the phenotypic difference between the parents and the F_1 hybrid, which rejected the possibility that light is involved in VSR expression. In two separate, pilot experiments, germination in 1 mM KCl solution increased the F_1 root growth, suggesting that K^+ participated in the VSR suppression in the deep pot conditions. Recent research in Arabidopsis found that K^+ deprivation strongly induced and accumulated ROS in root cells, which activated the Ca^{2+} channels. The elevated cytoplasmic Ca^{2+}, in turn, stimulates NADHP oxidase-mediated ROS production (reviewed by Wang and Wu 2010; Alemán et al. 2011). Furthermore, impairment of the vacuolar Ca^{2+} pumps ACA4 and ACA11 caused accumulation of ROS and activation of a salicylic acid-dependent PCD pathway in Arabidopsis (Boursiac et al. 2011). All this implies that ROS may be also involved in the VSR expression, possibly via the K^+ and Ca^{2+} signaling pathways.

In another aspect, supplement of KCl in tap water at different concentrations, however, did not change the F_1 root growth significantly, probably due to the counteraction by the bivalent cations such as Ca^{2+} and Mg^{2+} in the tap water. At the meantime, germination in 1 mM $CaCl_2$ or 1 mM $MgCl_2$ caused the F_1 roots even shorter. This suggests the bivalent-charged cations might promote the VSR and their effects were neutralized in the vermiculite condition. In Arabidopsis, several cyclic-nucleotide-gated ion channels (CNGC) involved non-selective uptake of K^+, Na^+ and Ca^{2+} and variation in the coding genes led to PCD, senescence, and lesion mimic (reviewed by Chin et al. 2009). Considering the complexity in mechanisms of ion competition, we will conduct rigouous physiological tests on a larger scale using isogenic F_1 hybrids. To this end, we transferred *Vsr1b* and the dominant male sterility gene *Ms3*, which is located on chromosome arm 5AS (Maan et al. 1987), into the CS background. Pollinating the male sterile CS-*Ms3* and CS-*Ms3*-*Vsr1b* with TA and CS will facilitate to produce sufficient seeds of two isogenic hybrid F_1 pairs for physiological characterization of VSR development and suppression.

VSR as a model for non-additive interaction study in wheat

Non-additive or epistatic interactions are intriguing mainly due to their transgressive effects on hybrid phenotypes. While many mechanisms have been proposed for transgressive inheritance in hybrid traits, recent studies showed that small RNAs, particularly miRNAs and siRNAs, are involved in governing transgression in Arabidopsis (Ha et al. 2009; Groszmann et al. 2011) and tomato (Shivaprasad et al. 2012). For systemically characterizing the molecular mechanisms of non-additive interactions underlying the hybrid weakness in wheat, a model system would be desirable. Unlike *Ne1/Ne2*-meidated hybrid necrosis, the VSR syndrome did not have severely adverse effects on shoot growth and the VSR plants completed their life cycle regularly. As a result, the VSR plants produce sufficient seeds for molecular and physiological studies. Other advantages of this VSR system include simple genetics of a single locus and convenience of phenotyping at a very early developmental stage in laboratory conditions. All this makes VSR an attractive model for the study of non-additive interactions and hybrid weakness. Molecular cloning of the *Vsr1* gene will be the first step to characterize its non-additive interaction. The *Vsr1* gene is located in the distal region of 5DL, where recombination level is much higher than the genome-wide average (Qi et al. 2004). In addition, high polymorphism levels and availability of a BAC-based physical maps in the D-genome donor species *Ae. tauschii* (Dvorak et al. 2012) strongly favors a map-based cloning approach. Bobwhite (BW), the highly transformable wheat cultivar, contains the *Vsr1a* allele (Table 1). We also transferred the *Vsr1b* allele into BW. This will facilitate functional validation of the *Vsr1* candidates by genetic transformation. With the true-breeding transgenic VSR plants, we will be able to identify VSR suppressors in CS and TA. Another aspect of investigation into the VSR mechanisms will be to profile the *Vsr*-dependent mRNA and small RNA transcriptomes and proteome. We have developed *Vsr1* near-isogenic lines in the BW, CS and TA backgrounds. They are the ideal materials for transcriptome and proteome profiling. A combination of these two approaches will provide us a better view of the non-additive interaction underlying the VSR phenotype in the context of root development.

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