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ORIGINAL PAPER

Addition of rye chromosome 4R to wheat increases anther length and pollen grain number

Vy Nguyen · Delphine Fleury · Andy Timmins · Hamid Laga · Matthew Hayden · Diane Mather · Takashi Okada

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Abstract

Key message **The research identified rye chromosome 4R arms associated with good pollinator traits, and demonstrated possible use of rye genetic resources to develop elite pollinators for hybrid wheat breeding.**

Abstract Bread wheat (*Triticum aestivum*) is a predominantly self-pollinating plant which has relatively small-sized anthers and produces a low number of pollen grains. These features limit the suitability of most wheat lines as pollinators for hybrid seed production. One strategy for improving the pollination ability of wheat is to introgress cross-pollination traits from related species. One such species is rye (*Secale cereale* L.), which has suitable traits such as high anther extrusion, long anthers containing large amounts of pollen and long pollen viability. Therefore, introducing these traits into wheat is of great interest in hybrid wheat breeding. Here, we investigated wheat–rye chromosome addition lines for the effects of rye chromosomes on anther and pollen development in wheat. Using a single nucleotide polymorphism genotyping array, we detected 984 polymorphic markers that showed expected syntenic relationships between wheat and rye. Our results revealed that the addition of rye chromosomes 1R or 2R reduced pollen fertility, while addition of rye chromosome 4R increased anther size by 16 % and pollen grain number by 33 %. The effect on anther length was associated with increases in both cell size and the number of endothecium cells and was attributed to the long arm of chromosome 4R. In contrast, the effect on pollen grain number was attributed to the short arm of chromosome 4R. These results indicate that rye chromosome 4R contains at least two genetic factors associated with increased anther size and pollen grain number that can favourably affect pollination traits in wheat.

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V. Nguyen · D. Fleury · A. Timmins · H. Laga · D. Mather · T. Okada (✉)
Australian Centre for Plant Functional Genomics and School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, Glen Osmond, SA 5064, Australia
e-mail: takashi.okada@acpfg.com.au

H. Laga
Phenomics and Bioinformatics Research Centre, University of South Australia, Mawson Lakes, SA 5095, Australia

M. Hayden
Department of Environment and Primary Industry, AgriBioSciences, La Trobe R&D Park, Bundoora, VIC 3083, Australia

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important crops for human nutrition and for feeding a growing world population. However, the scale of sustained increase in global food production is unprecedentedly high and requires substantial changes in methods for agronomic processes and crop improvement (Tester and Langridge 2010). Therefore, new breeding strategies such as the production and release of superior hybrid varieties have attracted great interest from the public sector and private industry with the aim of increasing annual yield in order to meet world demand (Whitford et al. 2013). Estimated yield improvement associated with hybrid vigour in wheat ranges from 5 to over 20 % (Boland and Walcott 1985;

Dreisigacker et al. 2005; Uddin et al. 1994). In a recent study, over 1,600 combinations of winter wheat hybrids showed superior traits and average of approximately 10 % increase in grain yield as well as resistance against biotic and abiotic stresses (Longin et al. 2013). For efficient F_1 hybrid seed production in wheat, it is important that the paternal parent has good pollination ability. Ideally, good pollinator plants of wheat would be tall with long extruded anthers producing large quantities of long-life pollen that is able to disperse metres away for cross-pollination (Whitford et al. 2013). In wheat itself, the genetic resources for such traits are currently limited (Langer et al. 2014).

Wheat is a predominantly self-pollinating plant species (Curtis and Johnston 1969; Pickett 1993), in which the anthers dehisce inside the florets before or just after flowers open. In contrast, rye (*Secale cereale* L.) is a cross-pollinating plant species, in which long anthers are extruded from the florets and shed large numbers of pollen grains (Lundqvist 1956). The length of wheat anthers has been reported to range from 2.99 to 4.41 mm (De Vries 1974; Kherde et al. 1967), while rye anthers can be as long as 8.25 mm (Athwal and Kimber 1970; Immonen and Anttila 1998). Anther size has been reported to be positively correlated with the number of pollen grains (Milohnic and Jost 1970; Pickett 1993). In rye, pollen production per spike (42×10^5 pollen grains) is roughly ten times greater than that in wheat (45×10^4 pollen grains) (Reddi and Reddi 1986) and the average pollen grain number per anther of rye is approximately 19,000 compared to 2910 in wheat (Waines and Hegde 2003). D'Souza (1970) reported that with moderate wind speed conditions, rye pollen could be carried about 70 m further and about 0.4 m higher than wheat pollen. Moreover, wheat pollen loses viability within 15–30 min after release, while rye pollen can remain viable for 72 h or more under optimal field conditions (D'Souza 1970; Fritz and Lukaszewski 1989).

Both wheat and rye belong to the tribe Triticeae of the Poaceae (grasses) family, and fertile hybrids can be generated between them, such as *Triticale* (×*Triticosecale* Witt). Rye has been used to improve wheat cultivars, mainly for disease resistance. For example, a segment of the short arm of rye chromosome 1R has been introduced into wheat to improve resistance against powdery mildew (*Blumeria graminis* f. sp. *tritici*) and stem, leaf and stripe rust (*Puccinia* spp.) (Friebe et al. 1996; Mago et al. 2004). In contrast, the use of rye genetic resources to improve wheat floral structure for hybrid seed production has not been thoroughly investigated. Wheat–rye chromosome addition lines, hereafter referred to as 'addition lines', have been developed and used as genetic resources and research tools to identify useful genetic traits in rye for wheat breeding (Driscoll and Sears 1971; Evans and Jenkins 1960; Riley and Chapman 1958). These include a set of disomic addition lines that carry individual pairs of chromosomes (1R–7R) or chromosome arms of Imperial rye in a Chinese Spring wheat background, and they revealed changes in spike and spikelet morphology (Driscoll and Sears 1971; Zeller and Hsam 1983). Athwal and Kimber (1970) investigated anther size, anther extrusion and pollen longevity in some of these 'Chinese Spring–Imperial rye' addition lines, but did not identify any with significantly longer anthers, greater anther extrusion or longer pollen viability than Chinese Spring wheat. In contrast, Plaha and Sethi (2000) reported that the addition of rye chromosome 4R significantly increased anther length in wheat (Plaha and Sethi 2000). Further research was needed to establish whether the addition of any rye chromosomes affects anther length, pollen grain number or pollen fertility in wheat.

In this study, 'Chinese Spring–Imperial rye' wheat–rye chromosome addition lines (Driscoll and Sears 1971) were investigated to assess the effects of rye chromosomes on anther and pollen development. We examined wheat spike morphology, anther size, pollen fertility and pollen grain number in addition lines. We found each of these traits to be affected by the addition of specific rye chromosomes or chromosome arms to wheat.

Materials and methods

Plant materials

The bread wheat variety Chinese Spring and nine wheat–rye disomic chromosome addition lines (Chinese Spring–Imperial rye) developed by Driscoll and Sears (1971) were used (Table 1). Plants were grown in a glasshouse at the University of Adelaide, South Australia, during two periods. During the first period (15 December 2012–30 April 2013), each line was represented by three plants grown together in one large pot (20 cm high, 15 cm diameter).

Table 1 Plant materials used in this study

Line	Abbreviation
Wheat cv. Chinese Spring	CS
Imperial rye	RI
Wheat–rye chromosome 1 addition line	1R
Wheat–rye chromosome 2 addition line	2R
Wheat–rye chromosome 3 addition line	3R
Wheat–rye chromosome 4 addition line	4R
Wheat–rye chromosome 4 short arm addition line	4RS
Wheat–rye chromosome 4 long arm addition line	4RL
Wheat–rye chromosome 5 addition line	5R
Wheat–rye chromosome 6 addition line	6R
Wheat–rye chromosome 7 addition line	7R

These plants were used for validation and identification of each rye chromosome and for preliminary morphological observation. During the second period (1 March–31 July 2013), a replicated experiment was conducted in temperatures that ranged from approximately 14–24 °C and the day length maintained at approximately 12–h with the use of supplemental artificial lighting. Initially, at least five seedlings were grown in individual small pots (15 cm high, 10 cm diameter) and leaf samples were taken at the three-leaf stage. DNA was extracted, and marker analysis was conducted as mentioned above. Plants confirmed to carry the expected rye chromosomes were transplanted to a large pot (one plant per pot). Pots were arranged in a randomised complete block design with three blocks.

Confirmation of maintenance of rye chromosome in addition lines

The presence of rye chromosomes addition in Chinese Spring wheat was checked by using molecular markers. DNA was extracted from leaf samples as described in Rogowsky et al. (1993). Previously published simple sequence repeat (SSR), sequence-tagged site (STS) or expressed sequence tag (EST)-based markers (Supplementary Table S1) were assayed using the following PCR conditions: 94 °C, 30 s for denaturation; 45–60 °C, 40 s for annealing; 72 °C, 40 s for extension. Amplicons were electrophoretically separated on 2.5 % agarose gel containing SYBR® Safe DNA Gel Stain (Life Technologies) and visualised under UV light.

DNA samples from Chinese Spring wheat, Imperial rye and each addition line were also assayed on the Illumina wheat 9K iSelect BeadChip array (Cavanagh et al. 2013). Single nucleotide polymorphism (SNP) allele clustering and genotype calling were performed using GenomeStudio version 2011.1 software (Illumina) with an algorithm generated for bread wheat (Cavanagh et al. 2013). As discussed by Wang et al. (2014), genotype calling for such arrays is more complicated for polyploids than for diploids. Comparisons among a hexaploid (wheat), a diploid (rye) and a set of aneuploids (addition lines) add further complexity. Initially, the data were checked for conformity to a normal distribution by randomly selecting a small number of markers and applying a Shapiro–Wilk's test (Shapiro and Wilk 1965) using the R statistical environment. Then, the data were analysed using GenomeStudio software version 2011.1 (Illumina Inc., USA) to generate normalised values of R and θ. These values were handled in two ways. Firstly, to capture all cases for which one addition line (or two, in the case of the 4R and 4RS or 4RL addition lines) provided an 'outlier' result relative to Chinese Spring wheat and the other addition lines. The outlier was identified by applying a Grubb's test implemented in the 'outliers' R package (Komsta 2011).

For each of the polymorphic markers identified, a biplot of R versus θ was visually examined to confirm the result and to select markers for which the result for the 'outlier' addition line was intermediate between those for Chinese Spring and Imperial rye. Secondly, a subset of markers for which the 'outlier' addition line had a distinctly different θ value (ignoring the R value) from Chinese Spring wheat was selected, as these seemed likely to exhibit wheat–rye polymorphism at the target SNP itself (vs. polymorphism in copy number and/or probe affinity). Thus, they were suitable for the development of individual marker assays. For each selected marker, primers were designed using Kraken™ software (LGC Limited, London, UK) to develop KASP™ assays (Supplementary Table S2). The resulting assays were applied to DNA samples of Chinese Spring, Imperial Rye and each addition line using an automated SNP-line system (LGC Limited, London, UK).

Bioinformatic analysis

The iSelect array probe sequences that showed specific polymorphism in one of the addition lines were mapped to the rye genome zipper file (Martis et al. 2013) in order to assign physical position in the rye genome. Briefly, wheat iSelect array probe sequences were used for BLASTn search against *Brachypodium* CDS (MIPS version 1.2; http://mips.helmholtz-muenchen.de/plant/brachypodium/) and rice CDS (MSU RGAP Release 7 sequences; http://rice.plantbiology.msu.edu/index.shtml) with the cut-off E-value of 1E−10. Searches were conducted within the rye genome zipper file for the *Brachypodium* and rice gene models with the highest similarity.

Gene ontology (GO) information for annotated genes in the 4R genome zipper file was obtained as follows. Rice gene model IDs in the 4R genome zipper file were converted into MSU gene model IDs. Where a rice gene model was not available, *Brachypodium* or *Sorgum* gene model sequences were used to obtain rice gene model ID by BLAST search against rice CDS in order to obtain GO information. GO Slim gene ontology information (http://geneontology.org/) was obtained for the rice gene model by using Rice Genome Annotation tools (http://rice.plantbiology.msu.edu/downloads_gad.shtml). The obtained GO list was filtered by selecting rice genes with putative function for cell differentiation (GO:0030154), cell growth (GO:0016049), cell cycle (GO:0007049) or flower development (GO:0009908).

Anther sample collection and pollen fertility test

Just prior to anthesis, mature anthers were collected from primary and secondary florets of the middle part of spikes, fixed in FAA (50 % ethanol, 5 % acetic acid, 4 %

formaldehyde) for overnight and then stored in 70 % ethanol at 4 °C. Three anthers were split open in iodine–potassium iodide (KI) solution (1 % iodine and 10 % potassium iodide). The pollen grains released from these anthers were observed under a microscope for the accumulation of starch. In this method, the starch that accumulates in fertile pollen grains stains darkly, while sterile pollen grains remain light in colour (Jefferies 1977). The remaining anthers were preserved in 70 % ethanol at 4 °C for the other experiments.

Observation of pollen by scanning electron microscopy

Anthers that had been preserved in 70 % ethanol were dehydrated using a sequence of 15-min incubations in 80, 90, 95 and 100 % dehydrated ethanol. Each dehydrated anther was cut in half and dried using a critical point dryer (Bal-tec CPD 030). Pollen grains from these anthers were mounted on studs and coated with carbon. Images of pollen grains and pollen pores were taken under the Philips XL20 Scanning Electron Microscope in the Adelaide Microscopy Facility (Urrbrae, Australia) with the condition of 10 kv beam, spot 3.

Preparation of semi-thin anther sections

Anthers that had been preserved in 70 % ethanol were rehydrated in a sequence of 15-min incubations in 50 % ethanol, 30 % ethanol and phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Each anther was trimmed at both ends and embedded in 2 % low-melting agarose in PBS. Agarose blocks containing anthers were dehydrated through an ethanol series for 15 min each (30, 50, 70, 85, 90, 95, 100 %) and two more times in 100 % ethanol. Technovit solution was prepared according to the manufacturer's instructions (Kulzer's Technovit 7100, Wehrheim, Germany). The samples were infiltrated in a sequence of 2-h incubations in ethanol–Technovit solution with proportion of 3:1, 1:1, 1:3 and finally 100 % Technovit solution for 3 times and left overnight. Each anther-containing agarose block was transferred into a 0.2-ml-thin-wall PCR tube with desired orientation and polymerised at room temperature. Each block was trimmed under a dissecting microscope, and 5-μm-thin longitudinal sections of anther were prepared using a Leica RM2265 rotary microtome. The sections were stained by 0.05 % toluidine blue solution (Soukup 2014) and mounted with DPX Mountant (Fluka Analytical, Switzerland).

Measurement of anther length and cell size

Images of preserved anthers (20 anthers × 3 plants for each line) were taken using a stereo dissecting microscope (Leica MZFL III), and images of pollen grains were taken using a Leica AS LMD microscope. Anther length was measured using image analysis software ImageJ (http://imagej.nih.gov/ij/index.html). Images of semi-thin anther longitudinal sections (7–8 sections for rye, CS, 4R, 4RL and 4RS, and 3–4 sections for other RCA lines) were taken under a Leica AS LMD microscope. The lengths of 20 endothecium cells were measured by ImageJ, and the average cell length was calculated.

Counting of pollen grains

Three anthers collected from each plant were transferred to a 1.5-ml centrifuge tube containing 500 μl water and 100 μl KI solution. These anthers were gently split open using a small pestle. The anther sample was then mixed vigorously by vortex for 1 min to release all pollen grains from the anthers to the solution. A drop of pollen suspension (15 μl) from the tube was pipetted onto a well of 96-well plate lid. A coverslip was placed on top of the pollen suspension, and images of pollen grains were taken under a stereo dissecting microscope (Leica MZFL III). There were at least three biological replicates for each line, and in each biological replicate, three to ten images were taken as technical replicates. The number of pollen grains in each image was counted using a MATLAB (MathWorks, MA, USA) programme written for this project. The standalone programme is available on request from the corresponding author. The mean number of pollen grains per anther was calculated.

Statistical analysis

Statistical analyses were performed using SPSS Statistics version 20.0 (IBM, NY, USA) by univariate general linear model to ensure the effect of block and spike on anther length and pollen grain number. One-way analysis of variance was used to examine differences between mean values of anther length, endothecium cell size and pollen grain number per anther with 9 planned single degree of freedom contrasts to compare each wheat–rye chromosome addition line with Chinese Spring wheat.

Results

Confirmation of rye chromosome addition in wheat

The wheat–rye (Chinese Spring–Imperial rye) chromosome addition lines used here were developed by Driscoll and Sears (1971) and had been maintained at the University of Adelaide for several generations. It was possible that rye chromosomes could have been lost during maintenance

Fig. 1 Representative images of spike (*a*), spikelet (*b*) and floret (*c*) from bread wheat cultivar Chinese Spring (CS), Imperial rye (RI) and wheat–rye chromosome addition lines (1R–7R). *Scale bars* in $a = 2$ cm, $b = 5$ mm, and $c = 2$ mm

(Alkhimova et al. 1999; Riley 1960; Szakács and Molnár-Láng 2010); thus, we first checked the genetic integrity of the materials. Nevertheless, some of the addition lines were clearly morphologically different from Chinese Spring wheat. For example, plants of the 2R addition line had slender spikes and their spikelet architecture differs from that of Chinese Spring wheat, while plants of the 5R addition line had crab-head-shape spikes (Fig. 1), as reported by Zeller and Hsam (1983). Other addition lines showed only minor variations in the size and morphology of spikes and spikelets, and were not clearly distinguishable from each other nor from Chinese Spring wheat. Therefore, we used molecular marker assays to check for the presence of the expected rye chromosome in each addition line.

Of 26 markers selected from previous reports on genetic mapping in rye (Nkongolo et al. 2009; Saal and Wricke 1999; Tomita et al. 2012; Xu et al. 2012), 15 exhibited PCR amplicons (Supplementary Figure S1 and Table S1) specific to the presence of particular rye chromosomes. These markers were used for selection and confirmation of plant materials for phenotyping. Subsequently, additional polymorphisms were detected using an Illumina iSelect 9K wheat array (Cavanagh et al. 2013) to verify the integrity of the added rye chromosome in representative plants for each addition line. Out of 8,632 markers on the array, there were 1,185 for which one of the addition lines could be readily distinguished from Chinese Spring and the other addition lines based on values of R and/or θ (Fig. 2). Of the 1,185 iSelect probes for which such deviations were observed, 984 had previously been positioned on a consensus genetic map of wheat (Cavanagh et al. 2013) and 411 could be mapped onto the virtual rye genome contig file Genome Zipper (Martis et al. 2013) based on sequence similarity of array probes with *Brachypodium* and rice sequences (Table 2). For example, 237 markers with positional information in the wheat genetic map exhibited a distinct result for the 1R addition line, and 83 of these mapped to chromosome 1R in the rye Genome Zipper. Among those, 204 markers had been positioned on a group 1 chromosome in the wheat consensus map and only 33 to other wheat chromosomes (Table 2). This is consistent with the expected synteny between rye chromosome 1R and the group 1 chromosomes of wheat. Similarly, syntenic relationships were observed between rye chromosome 4R and wheat chromosome groups 4, 6 and 7, and also for other chromosome combinations. This reflects chromosome rearrangements in Triticeae species during evolution and is largely consistent with the previous reports on wheat–rye synteny (Devos et al. 1993, 1995; Martis et al. 2013). Out of 42 markers selected for conversion to KASP assays, 24 were successfully converted to assays that clearly

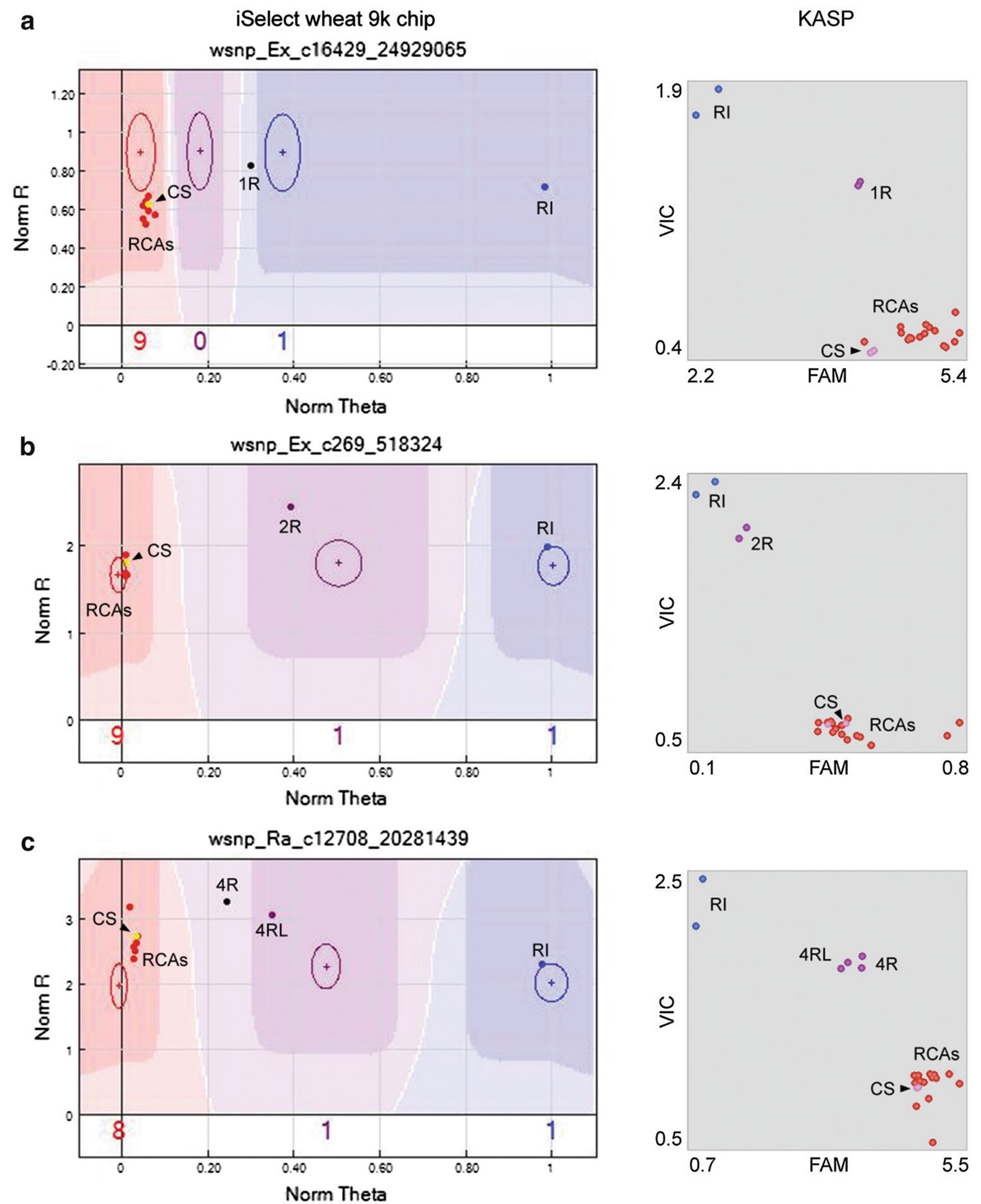

Fig. 2 Detection of polymorphism in specific wheat–rye chromosome addition lines by iSelect wheat 9K array and by KASP™ assays designed based on the corresponding array probe sequences. For each of the three markers shown, the normalised θ value (horizontal axis of the iSelect results shown in the *left*) of one specific addition line (*black dot*) is located between those of Chinese Spring (CS; *yellow dot*) and Imperial Rye (RI; *blue dot*), while those of the other wheat–rye chromosome addition line signals (RCAs; *red dots*) are near that of CS. With the KASP™ assays designed for these markers (*right*), the same specific addition lines (*purple dots*) are located between Chinese Spring (CS; *pink dots*) and Imperial Rye (RI; *blue dots*), and clearly separated from other addition lines (RCAs; *red dots*). In the KASP™ assay results, each line is represented by *two dots*, because the assays were applied to duplicated samples of each line. **a** Marker wsnp_Ex_c16429_24929065 for 1R. **b** Marker wsnp_Ex_c269_518324 for 2R. **c** Marker wsnp_Ra_c12708_20281439 for 4R and 4RL

Table 2 Number of iSelect wheat 9K array probe that distinguish specific wheat–rye chromosome addition lines from Chinese Spring wheat

Wheat chromosome group[a]	1R	2R	3R	4R	4RS	4RL	5R	6R	7R
1	204	4	2	1	0	1	1	0	5
2	3	171	4	2	0	3	0	0	15
3	21	6	78	1	0	1	1	21	3
4	6	2	4	22	10	11	7	1	45
5	1	1	1	0	0	0	88	2	12
6	0	3	7	13	1	13	1	78	2
7	2	13	0	34	34	44	2	6	39
Total	237	200	96	73	45	73	100	108	121
Rye genome[b]	(83)	(86)	(43)	(32)	(10)	(30)	(56)	(46)	(55)

[a] Physical location of 9K iSelect array probes in wheat chromosome groups

[b] Number of markers successfully mapped on the rye Genome Zipper file (Martis et al. 2013)

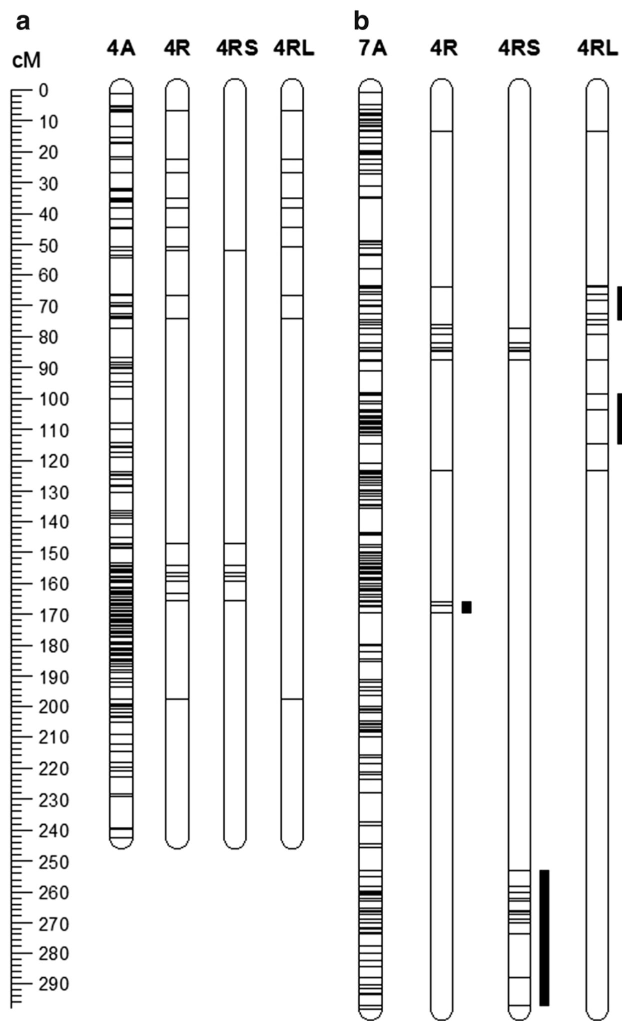

Fig. 3 Markers that distinguish the 4R chromosome or chromosome arm addition lines from wheat Chinese Spring. **a** Markers that had previously been mapped on wheat chromosome 4A. The *left diagram* represents wheat chromosome 4A with cM scale, and 9K array probes with known chromosome location are indicated by *crossbars* in the diagram. The other three diagrams indicate the positions of markers (*crossbars* on wheat chromosome 4A) that distinguish the 4R, 4RS and 4RL addition lines from Chinese Spring wheat. **b** Markers that had previously been mapped on wheat chromosome 7A and distinguish the 4R, 4RS and 4RL addition lines from Chinese Spring wheat. *Vertical black bars* indicate regions of wheat chromosome 7A, in which there were multiple markers that distinguished only one of the three addition lines from Chinese Spring wheat

distinguished one addition line from the other addition lines, from Chinese Spring and from Imperial Rye (Fig. 2 and Supplementary Table S2).

Putative chromosome rearrangements found in 4R, 4RS and 4RL addition lines

It is important to check genomic organisation of chromosome arm addition lines as alien chromosome is often unstable due to the role of gametocidal genes and preferential transmission or elimination of certain chromosomes (Jiang et al. 1994a). Using information on polymorphic iSelect markers, we examined the composition of the rye chromatin in the 4R, 4RS and 4RL addition lines. Consistent with wheat–rye synteny (Martis et al. 2013), the 4R addition line mainly included markers from wheat chromosome groups 4, 6, 7 (Table 2). One would expect that the markers distinguishing 4RS or 4RL from Chinese Spring would also distinguish 4R from Chinese Spring. This was the case for most of the markers that had previously been mapped on wheat chromosome groups 1 through 6. For example, of 20 markers that had previously been mapped on wheat chromosome 4A and that differentiated the 4R addition line from Chinese Spring, 8 were detected for both 4R and 4RS, 11 for both 4R and 4RL and only one for 4R only (Fig. 3a).

In contrast, among 34 markers that had been mapped on wheat group 7 chromosomes and that distinguished the 4RS addition line from Chinese Spring, only 7 markers distinguished the 4R addition line from Chinese Spring (Table 2). Similarly of 44 group 7 markers detected for 4RL, only 24 were also detected for 4R. There were also three markers that were detected for 4R but not for either 4RS or 4RL. Figure 3b clearly shows that sets of markers closely located in particular part of wheat chromosome 7A (indicated by vertical black bars) were uniquely present in 4RS or 4RL chromosome arm addition lines. Based on synteny information between rye chromosome 4R and wheat chromosome 7A (Martis et al. 2013), a unique segment of wheat chromosome 7A identified in 4RS (black bar in 4RS in Fig. 3b) is expected to be absent, while unique segments identified in 4RL are expected to be present in 4R. These suggest the loss of chromosome segment syntenic to wheat chromosome group 7 in the 4R addition line and addition or rearrangement of chromosome segment syntenic to wheat chromosome group 7 in the 4RS addition line during the process of resource development or maintenance.

Addition of the long arm of rye chromosome 4R increases anther length in wheat

Differences in anther length among addition lines were evident from morphological observations of florets (Fig. 1c). In Chinese Spring wheat, the mean anther length was 3.85 ± 0.14 mm, while anther lengths of the addition lines ranged from 2.66 ± 0.26 to 4.55 ± 0.28 mm (Table 3). Among the addition lines, the 1R and 2R lines had the shortest anthers (only 2.88 ± 0.21 and 2.66 ± 0.26 mm, respectively), while the 4R and 4RL lines had the longest anthers (4.45 ± 0.26 and 4.55 ± 0.28 mm, respectively). Therefore, it is clear that the long arm of rye chromosome 4R carries a major locus or loci that can increase anther

Table 3 Anther and endothecium cell length and pollen grain number in wheat–rye chromosome addition lines

Plant	Anther length (mm)	Endothecium cell length (μm)	Pollen grains/anther
CS	3.85 ± 0.14	26.9 ± 2.2	2912 ± 202
1R	2.88 ± 0.21*	22.7 ± 2.3	1867 ± 94*
2R	2.66 ± 0.26*	24.5 ± 3.3	2080 ± 173*
3R	3.78 ± 0.20	23.2 ± 2.5	2788 ± 677
4R	4.45 ± 0.26*	29.6 ± 0.9	3859 ± 113*
4RS	4.05 ± 0.20*	27.5 ± 2.4	3684 ± 61*
4RL	4.55 ± 0.28*	28.6 ± 2.0	3381 ± 351
5R	3.95 ± 0.28	25.8 ± 0.9	3367 ± 378
6R	3.90 ± 0.16	24.2 ± 2.8	3117 ± 242
7R	3.65 ± 0.24*	25.5 ± 3.9	2323 ± 250

* Significantly different from Chinese Spring sample, $p < 0.01$ (t test)

length in wheat. The anthers of the 4RS addition line were slightly longer than those of Chinese Spring (Table 3), indicating that the short arm of chromosome 4R may have loci with minor effects on anther length.

Addition of 1R and 2R rye chromosomes reduces pollen fertility in wheat

Given that male-sterile plants often have small anthers (Laser and Lersten 1972; Saini et al. 1984), we examined variation on pollen fertility among the wheat–rye addition lines. First, we checked the structure of round-shape (presumably fertile) pollen by scanning electron microscopy and confirmed that there is no obvious difference in pollen structural and surface architecture among the addition lines (Supplementary Figure S2). Next, we investigated the degree of pollen fertility by using KI staining to confirm starch accumulation in mature pollen grains. The pollen grains from Chinese Spring wheat and from most of the addition lines stained darkly, indicating high levels of starch accumulation and possibly fertile pollen, while those from the 1R and 2R addition lines pollen were pale brown, indicating a lack of starch accumulation and sterility (Fig. 4, left panel). For the 2R addition line, we observed differences in degree of sterility between two seasons in which we grew the addition lines. Pollen samples collected from plants grown in summer (January–April 2013 in Adelaide, Australia) exhibited partial sterility, while those collected from plants grown in autumn (March–June 2013) exhibited complete sterility (Fig. 4). Thus, the 2R chromosome may carry a genetic factor that negatively affects pollen fertility, depending on growth conditions, possibly temperature and photoperiod. Further investigation using semi-thin sections of anthers confirmed the lack of cytoplasmic components in the pollen from the 2R addition line (Fig. 4, right panel). Consistent with the reduced pollen fertility, the 2R addition line had poor seed set. Defective pollen and partial sterility were also observed for the 1R addition line (Fig. 4), but not for the 3R, 4R, 4RS, 4RL, 5R, 6R or 7R addition lines. Therefore, the 1R and 2R chromosome may carry loci that negatively affect pollen fertility, causing significant reduction in pollen fertility and anther length in these lines.

Addition of rye chromosome 4R increases the number of pollen grains in wheat anthers

Changes in anther length among addition lines could be due to changes in cell size or cell number or both in anther walls. To investigate whether differences in anther length involve these factors, cell length was measured for the endothecium layer beneath the epidermis of anthers (Fig. 4, white arrow head in the right panel). Endothecium cell length ranged from 22.7 ± 2.3 (the 1R addition line) to 29.6 ± 0.9 μm (the 4R addition line) (Table 3), and there seemed to be some positive association between endothecium cell length and anther length ($r = 0.76$, Supplementary Figure S3a). Thus, large anther size in 4R and 4RL is partly due to the expansion of endothecium cells in anther wall.

Given that anther length had been reported to be positively correlated with pollen grain number among and within the wheat varieties (De Vries 1974; Milohnic and Jost 1970; Pickett 1993), we investigated the association between these traits among Chinese Spring wheat and the wheat–rye addition lines. We observed a strong positive correlation ($r = 0.93$) between mean anther length and the mean number of pollen grains per anther (Supplementary Figure S3b). The short-anther 1R and 2R addition lines contained significantly fewer pollen grains per anther than Chinese Spring wheat (Table 3), and the long-anther 4R addition line contained significantly more pollen grains per anther than Chinese Spring wheat. Despite having anthers that were only slightly longer than those of Chinese Spring wheat, the 4RS line had almost as many pollen grains per anther as the 4R addition line. These results indicate that the increase in pollen grain number conferred by chromosome 4R is mainly associated with the short arm of that chromosome, in contrast to the increase in anther length, which is associated with the long arm.

Discussion

Wheat lines with chromosome additions, introgressions or translocations from related species (such as rye, barley, *Elymus trachycaulus*, *Aegilops speltoides*, *Ae. ventricosa*, *Thinopyrum intermedium*, *Th. ponticum*) are important resources to introduce genetic diversity for wheat breeding.

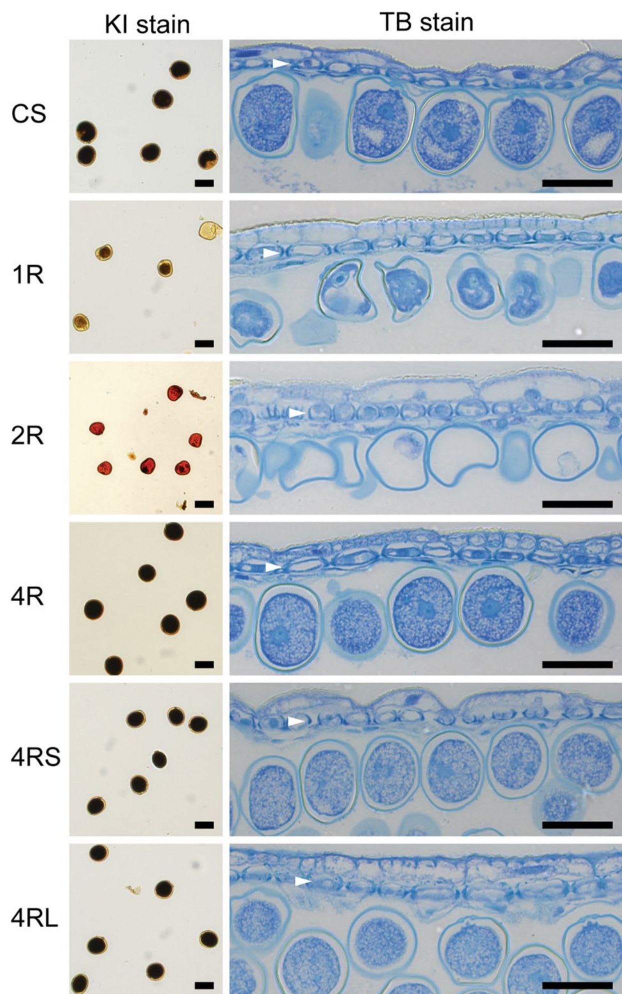

Fig. 4 Pollen fertility assessment by iodine–potassium iodide (KI) staining and morphological observation. *Dark colour* pollen grains stained by KI indicate starch-filled fertile pollen, *while pale brown* pollen grains do not have accumulated starch and are sterile (*left*). Longitudinal sections of mature anthers were stained by toluidine *blue* (TB stain, *right*). Endothecium cells in the anther wall are indicated by *white*. *Scale bars* in all the panels = 50 µm

Historically, confirmation of the presence of the alien chromatin in such lines used cytogenetic approaches (chromosome counting, C-banding, GISH and FISH) and/or individual molecular markers (Badaeva et al. 2008; Cseh et al. 2011; Deng et al. 2013; Jiang et al. 1994b; Li and Wang 2009; Sakai et al. 2009; Szakács and Molnár-Láng 2010). The cytogenetic methods generally require extensive skills and experience to obtain reliable information, whereas individual genetic markers provide limited genomic and genetic information. In this study, we have demonstrated that an Illumina iSelect array designed for wheat (Cavanagh et al. 2013) can be used for molecular analysis of wheat–rye addition lines. About a thousand markers from 9K wheat iSelect array were found to be useful for detecting specific rye chromosomes added to wheat. The distribution of these markers for each wheat–rye addition line on a wheat consensus map showed syntenic relationships between rye and wheat chromosomes as expected. While some of the polymorphisms detected with the array may reflect copy number variation due to the addition of a rye chromosome, others reflect wheat–rye SNPs. This was confirmed by the successful design of KASP™ assays for some of these SNPs. These assays, as well as SSR assays that were tested here, should be useful for monitoring specific rye chromosomes in a Chinese Spring wheat background. We also found some unexpected results in addition lines that carry one or both arms of chromosome 4R, with more polymorphisms detected for the 4RS and 4RL addition line than for the 4R addition line, suggesting chromosome rearrangement. To refine the position of chromosomal rearrangement, these lines could be assayed on more highly multiplexed genotyping platforms such as the 90K wheat array (Wang et al. 2014).

We have shown that the addition of rye chromosomes to wheat can substantially affect floral architecture and pollen fertility. Chromosomes 2R and 5R affected spike and spikelet morphology, while chromosomes 1R, 2R and 4R affected anther and pollen development. Increases in anther length were attributed to the long arm of chromosome 4R, while increases in pollen grain number were attributed to the short arm of that chromosome. This indicates that rye 4R chromosome contains genetic factors associated with anther and pollen grain development and demonstrated that a rye chromosome arm can sufficiently change wheat anther traits. The effects observed here for anther length differed from those reported by Athwal and Kimber (1970) in ‘Chinese Spring–Imperial rye’ wheat–rye chromosome addition lines, but they have some similarities to those reported by Plaha and Sethi (2000). Differences in the results obtained in the three investigations may reflect the effects of growth and environmental conditions on anther size.

The substantial increase in pollen grain number (33 %) associated with the addition of rye chromosome 4R to wheat could provide a basis for improving one of the key traits needed in pollinator parents for hybrid wheat (Pickett 1993). The same chromosome has been reported to considerably increase anther culture responses and callus formation (Lazar et al. 1987). Perhaps rye chromosome 4R carries genetic factors that contribute cell growth and the cell cycle both in vitro and in vivo. Among genes that are known to affect the development of anthers and pollen in flowering plants (Zhang et al. 2011), *GAMYB* and other R2R3-type *MYB* genes are known to influence gene expression and reduce pollen fertility in rice and Arabidopsis, respectively (Aya et al. 2009; Dubos et al. 2010). There are also numbers of genes that affect pollen development and fertility in crops (Chen and Liu 2014; Guo and Liu

2012), resulting in smaller size of anther and induced sterility (Laser and Lersten 1972; Saini et al. 1984). But to date, there is no gene identified for increasing anther size and increased pollen grain number. In maize, co-suppression or silencing of the *Cell Number Regulator1* (*CNR1*) gene is known to increase plant and organ size through increased cell number, but it is not known whether this gene affects anther size or pollen grain number (Guo et al. 2010). It would be interesting to investigate the expression level of wheat and rye *CNR1* orthologues in the anthers of the 4R addition line. It would also be interesting to investigate the transcriptional profiling of rye genes that are known to be on chromosome 4R in order to identify pathways that could be associated with variation in anther traits (Martis et al. 2013). Genes with functional annotation related to the cell cycle, cell growth, cell differentiation and floral development (Supplementary Table S3) are of particular interest.

The work reported here involved a set of addition lines, in which pairs of chromosomes (or chromosome arms) from Imperial rye had been added to Chinese Spring wheat. By studying other sets of wheat–rye chromosome addition lines such as 'Holdfast-King II' (Riley and Chapman 1958) and 'Kharkov-Dakold' (Evans and Jenkins 1960), it would be possible to investigate whether anther and pollen development is affected by the source of the rye chromosomes and/or by the interactions with the wheat cultivars to which they are added. To assign the effects of rye chromosome 4R regions, it would be useful to introgress small segments of 4R chromatin into the wheat genome. While rye and wheat chromosomes do not naturally recombine with each other, using the wheat *ph1* mutant enabled us to induce recombination as demonstrated for a region on chromosome 1R (Anugrahwati et al. 2008; Rogowsky et al. 1993). By using this *Ph1*-mediated breeding method (Moore 2014), the Wheat Improvement Strategic Programme in the UK (http://www.wheatisp.org) is currently developing a large collection of introgression lines with *T. urartu*, *Th. bessarabicum*, *Ae. speltoides* and rye. These genetic resources would enable us to identify loci associated with increased anther length and pollen grain number. Then, the introduction of rye alleles into wheat could help to increase anther length and pollen grain number and to generate elite pollinator lines for hybrid wheat breeding.

Author contribution statement TO and DF designed and directed the project. VN grew plants and performed all the laboratory-based experiments. HL developed pollen counting imaging software and analysed image data. AT, MH, TO, DM analysed wheat 9K iSelect data. VN wrote first draft of the manuscript, and DM, DF, TO provided extensive editing to the manuscript. All authors reviewed the manuscript.

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Conflict of interest The authors declare no conflict of interest.

Ethical standard The authors note that this research is performed in accordance with ethical standards of the scientific conduct.

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