*Crop & Pasture Science*, 2013, **64**, 865–873 http://dx.doi.org/10.1071/CP13246

# Fast identification of wheat 1BL.1RS translocation by reversed-phase ultra-performance liquid chromatography (RP-UPLC)

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**Abstract.** The 1BL.1RS chromosomal translocation in wheat is the result of replacement of the short arm of chromosome 1B of wheat by the short arm of chromosome 1R of rye, which had been widely used as a parental line in worldwide wheat breeding, resulting in a high percentage of wheat cultivars containing this translocation. A fast and reliable approach to identify this translocation is highly desirable in modern wheat breeding. This study compared reversed-phase ultraperformance liquid chromatography (RP-UPLC), acidic polyacrylamide gel electrophoresis (A-PAGE), liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), allelic-specific PCR, and reversed-phase high-performance liquid chromatography (RP-HPLC) approaches to identify the 1BL.1RS translocation in 76 bread wheat cultivars. Two gliadin bands in the Gli-B1 region of A-PAGE separation were confirmed by LC-MS/MS to be omega secalins from the 1BL.1RS translocation, and they can be used as reliable protein markers for identifying the translocation. A few specific minor peaks eluted at 12–13 min on the RP-UPLC patterns can readily differentiate the 1BL.1RS translocation. Of the 76 wheat cultivars tested, 40 were identified as carrying the 1BL.1RS translocation by RP-UPLC, which was consistent with the results of A-PAGE, HPLC, and PCR. Compared with other established methods, RP-UPLC showed a clear advantage in fast identification of the 1BL.1RS translocation in wheat breeding.

Additional keywords: wheat, 1BL.1RS translocation, A-PAGE, HPLC, PCR, UPLC.

Received 10 July 2013, accepted 12 September 2013, published online 6 November 2013

# Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops and is the main food source for >40% of the world population. The 1BL.1RS translocation in wheat resulted from replacing the short arm of the chromosome 1B of wheat by the short arm of the chromosome 1R of rye (*Secale cereale* L.). In the past few decades, this translocation had been widely used in wheat breeding for improving disease resistance and yield performance (Zhao *et al.* 2012). Thus, the short arm of rye chromosome 1R is one of the most widely utilised alien chromatins in wheat breeding (Baum and Appels 1991). To date, ~300 cultivars and breeding lines carrying 1BL.1RS have

been released worldwide, mostly since 1970 (Graybosch 2001). About 50% of bread wheat cultivars released in China (Zhou *et al.* 2003), 55% in Bulgaria (Landjeva *et al.* 2006), and 53% in Hungary (between 1978 and 1999; Kószegi *et al.* 2000) carry the 1BL.1RS translocation. The translocation is also found in cultivars or germplasm produced in USA (Lukaszewski 1990), and Eastern Europe and Mexico (Villareal *et al.* 1997).

The 1BL.1RS translocation was initially designed to improve wheat resistance to several pathogens such as *Puccinia recondita*, *Puccinia graminis*, *Puccinia striiformis*, and *Blumeria graminis* induced by the genes *Sr31*, *Lr26*, *Yr8*, and *Pm8* located on 1RS (Zeller 1973). Cultivars carrying this translocation have

**Abbreviations:** ACN, Acetonitrile; A-PAGE, acidic polyacrylamide gel electrophoresis; AS-PCR, allelic-specific polymerase chain reaction; 2-DE, two-dimensional electrophoresis; HPCE, high performance capillary electrophoresis; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; LMW-GS, low molecular weight glutenin subunits; RP-HPLC, reversed-phase high-performance liquid chromatography; RP-UPLC, reversed-phase ultra-performance liquid chromatography; TFA, trifluoroacetic acid.

also shown other agronomic benefits, including higher yield and better yield stability (Zeller and Hsam 1983; Lelley et al. 2004). However, the 1BL.1RS translocation has been found to have detrimental effects on wheat end-use qualities, such as reduced gluten strength (diminished mixing tolerance) and loaf volume per unit protein, and increased dough stickiness during mixing (Dhaliwal et al. 1988; Milovanovic et al. 1998). These detrimental effects result from the introgression of  $\omega$ -secaling encoded by Sec-1 locus on the short arm of rve chromosome 1RS, and the loss of low molecular weight glutenin subunits (LMW-GS) encoded by Glu-B3 and gliadins encoded by Gli-B2 on the short arm of wheat chromosome 1BS (Javornik et al. 1991). Due to the negative effects on quality, breeders now seek to reduce the size of translocation in cultivars. A cost-effective and high-throughput approach is highly desirable for parental line selection and early-generation screening during quality wheat breeding.

So far, various methods have been developed to identify the wheat 1BL.1RS translocation, and the most widely used method is acidic polyacrylamide gel electrophoresis (A-PAGE; Koebner and Shepherd 1986). Isoelectric focusing (Chojecki et al. 1983), two-dimensional electrophoresis (2-DE, Gobaa et al. 2007), disease resistance tests (Heun and Fischbeck 1987; Friebe et al. 1989), and morphological observation of chromosomes (Zeller 1973; Mettin et al. 1973; Bennett and Smith 1975; Lukaszewski and Gustafson 1983; Rayburn and Carver 1988) are also among the methods for the identification of this translocation. However, these methods usually have disadvantages such as being time-consuming and having high costs, and therefore are not suitable for large-scale identification and screening in the early generations of wheat breeding. More recently, diagnostic techniques were developed such as ryespecific DNA probes (Gustafson et al. 1988; Heslop-Harrison et al. 1990), monoclonal antibodies (Howes et al. 1989), reversed-phase high-performance liquid chromatography (RP-HPLC, Lookhart et al. 1991), and high performance capillary electrophoresis (HPCE; Lookhart et al. 1996). However, these methodologies are sophisticated, with higher analysis costs, and therefore have not been used widely.

Reversed-phase ultra-performance liquid chromatography (RP-UPLC) is a new technology for the separation and characterisation of proteins based on the differences in surface hydrophobicity. It was developed based on RP-HPLC (Wu *et al.* 2006). In this system, small-diameter columns (e.g.  $1.7 \,\mu$ m) can be used and high column performance (up to 100 000–300 000 theoretical plates/m) can be produced. Compared with RP-HPLC, RP-UPLC analysis is nine times faster, two times higher in resolution, and three times more sensitive (Swartz 2005). Our recent study also showed that RP-UPLC is capable of separating wheat water-soluble proteins and LWM-GS with higher efficiency and resolution than traditional RP-HPLC (Yu *et al.* 2013*a*, 2013*b*).

In this study, an RP-UPLC method for rapid identifying the wheat 1BL.1RS translocations was developed. It was compared with A-PAGE, RP-HPLC, PCR, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods to aid the development of the RP-UPLC method. Our results demonstrate that RP-UPLC is capable of fast separation and identification of the secalins translocated from rye with high

resolution and reproducibility and lower costs, and thus has the potential for wide application in wheat breeding.

## Materials and methods

#### Wheat materials

Seventy-six common wheat cultivars (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) and one rye cultivar (*Secale cereale* L., 2n = 2x = 14, RR), which were mostly released and cultivated in China in the past two decades, were used in the current study (Supplementary Materials Table 1, as available on journal's website). All germplasm was collected from the National Wheat Germplasm Bank in the Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), China.

## Protein extraction

Gliadins were extracted from wheat flour by 70% ethanol. About 100 mg of flour was incubated in 400  $\mu$ L of 70% ethanol and oscillated at least 1 h, and then centrifuged at 13000 rpm for 15 min. The supernatant was transferred to two new 1.5-mL tubes, and loading buffer with 80% glycerol and 0.02% methyl chloride was added to one tube for A-PAGE. The other tube was centrifuged at 4°C and 13 000 rpm for 15 min, then the supernatant was transferred to a sample tube for RP-UPLC analysis.

# A-PAGE

The A-PAGE was performed by a modified method according to Yan *et al.* (2003), with 10% running gel and 5% stacking gel on Hoefer vertical electrophoretic apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA). Electrophoresis was carried out at a constant voltage of 300 V for 30 min, followed by 500 V for 4 h, at a constant temperature of 14°C. After electrophoresis, gels were stained with a mixture of 12.5 mL of 10% Coomassie Blue R dissolved in ethanol and 500 mL of 10% trichloroacetic acid solution, and destained with distilled water for 12 h.

#### LC-MS/MS

The expected bands were excised from A-PAGE gels and put in microcentrifuge tubes, and then washed with 200–400  $\mu$ L of 100 mM NH<sub>4</sub>HCO<sub>3</sub> and 30% acetonitrile (ACN). This step was repeated two or three times until the pellets were colourless, then the destaining buffer was removed and lyophilised for 30 min until the white particles became agglomerated. The dried particles were digested with 7  $\mu$ L of diluted trypsin solvent (Promega, Madison, WI, USA), diluted with 25 mM NH<sub>4</sub>HCO<sub>3</sub> to a final concentration of 15 ng/ $\mu$ L, and incubated at 37°C for at least 20 h. The peptides were extracted three times with 5% trifluoroacetic acid (TFA), 50% ACN, and 45% water at 37°C. Extracts were pooled and lyophilised, and stored at –80°C before mass spectrometric analysis.

The lyophilised, trypsin-digested peptides were dissolved in 0.1% TFA and mixed with 1  $\mu$ L of TFA, 500  $\mu$ L of ACN solution, and 499  $\mu$ L of water. The peptide mixtures were analysed using reversed-phase capillary liquid chromatography (LC) coupled with electrospray ionisation quadrupole time-of-flight (ESI-Q-TOF) tandem mass spectrometry (MS/MS) (LTQ VELOS; Thermo Finnigan, San Jose, CA, USA). The peptides were subsequently eluted onto a 0.15 mm by 150 mm analytical

Protein bands     PepCount sequence     Unique     Date     Accession     Start     End     Identified protein     Cover     MW <sup>C</sup> on A-PAGE <sup>A</sup> PepCount <sup>B</sup> Bank     number     (%)     (Da)       a     R.PFGQ0PEQIISQR.P     2     NCBI     ACQ83636.1     20     39     Omega secalin ( <i>Secale cereale</i> L.)     8.68     41607.12     3       b     R.PLFQLVQGQGIIQPQQPAQLEVIRS     1     NCBI     ACX37111.1     234     260     Gliadin ( <i>Triticum aestivum</i> L.)     8.47     33661.5     8       c     R.QLNPSEQELQSPQQPVPK.E     1     NCBI     ACN9689.1     20     39     Omega secalin ( <i>Secale cereale</i> L.)     7.05     27837.63     3	Protein bands       PepCount sequence       Unique       Date       Accession       Start       End       Identified protein       Cover       MW <sup>C</sup> on A-PAGE <sup>A</sup> PepCount <sup>B</sup> Bank       number       2       NCBI       Accession       Start       End       Identified protein       (%)       (Da)         a       R.PFGQ0PEQIISQR.P       2       NCBI       ACQ83636.1       20       39       Omega secalin ( <i>Secale cereale</i> L.)       8.68       41607.1.         b       R.PLFQLVQGQGIIQPQQPAQLEVIRS       1       NCBI       ACX37111.1       234       260       Gliadin ( <i>Triticum aestivum</i> L.)       8.47       33661.5         c       R.QLNPSEQELQSPQQPVPK.E       1       NCBI       ACN96898.1       20       39       Omega secalin ( <i>Secale cereale</i> L.)       7.05       27837.61											
on A-PAGE <sup>A</sup> PepCount <sup>B</sup> Bank         number         (%)         (Da)           a         R.PFGQ0PEQIISQR.P         2         NCBI         ACQ83636.1         20         39         Omega secalin ( <i>Secale cereale</i> L.)         8.68         41607.12         1           b         R.QLNPSEQELQSPQQPVPK.E         2         NCBI         ACX37111.1         234         260         Gliadin ( <i>Triticum aestivum</i> L.)         8.47         33661.5         8           c         R.QLNPSEQELQSPQQPVPK.E         1         NCBI         ACN9698.1         20         39         Omega secalin ( <i>Secale cereale</i> L.)         8.47         33661.5         8	on A-PAGE <sup>A</sup> PepCount <sup>B</sup> Bank number (%) (Da) R.PFGQQPEQIISQR.P R.PFGQQPEQIISQR.P R.QLNPSEQELQSPQQPVPK.E R.QLNPSEQELQSPQPVPK.E R.QLNPSEQELQSPQQPVPK.E R.QLNPSEQELQSPQPVPK.E R.QLNPSEQELQSPQPVPK.E R.QLNPSEQELQSPQPVPK.E R.QLNPSEQELQSPQPVPK.E R.QLNPSPVPK.E R.QNPVPK.E R.QLNPSVPK.E R.QLNPSPVPK.E R.QNPVPK.E R.QNPVVPK.E R.QNPVPK.E R.QNPVVPK	Protein bands	PepCount sequence	Unique	Date	Accession	Start	End	Identified protein	Cover	MW <sup>C</sup>	p/ <sup>D</sup>
a         R.PFGQQPEQIISQR.P         2         NCBI         ACQ83636.1         20         39         Omega secalin (Secale cereale L.)         8.68         41607.12         3.68           b         R.QLNPSEQELQSPQQPVPK.E         256         270         256         270         8.47         33661.5         3           b         R.RPLFQLVQGQGIIQPQQPAQLEVIRS         1         NCBI         ACX37111.1         234         260         Gliadin (Triticum aestivum L.)         8.47         33661.5         8           c         R.QLNPSEQELQSPQQPVPK.E         1         NCBI         ACN96898.1         20         39         Omega secalin (Secale cereale L.)         7.05         27837.63         2	a     R.PFGQQPEQIISQR.P     2     NCBI     ACQ83636.1     20     39     Omega secalin (Secale cereale L.)     8.68     41607.1       b     R.QLNPSEQELQSPQQPAQLEVIRS     1     NCBI     ACQ83636.1     256     270     8.68     41607.1       b     R.PLFQLVQGQGIIQPQQPAQLEVIRS     1     NCBI     ACX37111.1     234     260     Gliadin (Triticum aestivum L.)     8.47     33661.5       c     R.QLNPSEQELQSPQQPVK.E     1     NCBI     ACN96898.1     20     39     Omega secalin (Secale cereale L.)     7.05     27837.6.2	on A-PAGE <sup>A</sup>		PepCount <sup>B</sup>	Bank	number				(%)	(Da)	
R.QLNPSEQELQSPQQPVPK.E     256     270       b     R.PLFQLVQGQGIIQPQQPAQLEVIRS     1     NCBI     ACX37111.1     234     260     Gliadin (Triticum aestivum L.)     8.47     33661.5     8       c     R.QLNPSEQELQSPQQPVPK.E     1     NCBI     ACN96898.1     20     39     Omega secalin (Secale cereale L.)     7.05     27837.63     2	b     R.QLNDSEQELQSPQQPVPK.E     256     270       b     RRPLFQLVQGQGIIQPQQPAQLEVIRS     1     NCBI     ACX37111.1     234     260     Gliadin ( <i>Triticum aestivum</i> L.)     8.47     33661.5       c     R.QLNPSEQELQSPQQPVPK.E     1     NCBI     ACX37111.1     234     260     Gliadin ( <i>Triticum aestivum</i> L.)     8.47     33661.5       c     R.QLNPSEQELQSPQQPVPK.E     1     NCBI     ACN96898.1     20     39     Omega secalin ( <i>Secale cereale</i> L.)     7.05     27837.6:	a	R.PFGQQPEQIISQR.P	2	NCBI	ACQ83636.1	20	39	Omega secalin (Secale cereale L.)	8.68	41607.12	5.76
b     RRPLFQLVQGQGIIQPQQPAQLEVIRS     1     NCBI     ACX37111.1     234     260     Gliadin (Triticum aestivum L.)     8.47     33661.5     8       c     R.QLNPSEQELQSPQQPVPK.E     1     NCBI     ACN96898.1     20     39     Omega secalin (Secale cereale L.)     7.05     27837.63     27837.63	b     RRPLFQLVQGQGIIQPQQPAQLEVIRS     1     NCBI     ACX37111.1     234     260     Gliadin (Triticum aestivum L.)     8.47     33661.5       c     R.QLNPSEQELQSPQQPVPK.E     1     NCBI     ACN96898.1     20     39     Omega secalin (Secale cereale L.)     7.05     27837.6:		R.QLNPSEQELQSPQQPVPK.E				256	270				
c R.QLNPSEQELQSPQQPVPK.E 1 NCBI ACN96898.1 20 39 Omega secalin (Secale cereale L.) 7.05 27837.63 2	c R.QLNPSEQELQSPQQPVPK.E 1 NCBI ACN96898.1 20 39 Omega secalin ( <i>Secale cereale</i> L.) 7.05 27837.67	$^{p}$	RPLFQLVQGQGIIQPQQPAQLEVIRS	1	NCBI	ACX37111.1	234	260	Gliadin (Triticum aestivum L.)	8.47	33661.5	8.43
		С	R.QLNPSEQELQSPQQPVPK.E	1	NCBI	ACN96898.1	20	39	Omega secalin (Secale cereale L.)	7.05	27837.63	5.65

Molecular mass of the predicted protein. Proelectric point of the predicted protein. RP-C18 column (Column Technology Inc., Lombard, IL, USA) and separated at  $1 \,\mu$ L/min with an increasing ACN gradient from 4% to 50% from 0 to 30 min and from 50% to 100% at 34 min, then maintained to 40 min. The mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in ACN, respectively. The mass spectrometer was operated in a positive ion mode with a source temperature of 200°C and a cone gas flow of 10 L/h. All MS/MS spectra were processed using Mass Lynx 4.0 Protein Lvnx (Waters Corporation, Milford, MA, USA) to generate PKL files. The MS/MS spectra were searched in the NCBI (www.ncbi. nlm.nih.gov/) non-redundant green plant database (updated to 30/7/2012) using MASCOT version 2.1. The peptide tolerance was set to 100 ppm and fragment mass tolerance to 0.2 Da, two missed cleavages were allowed. Carbamidomethyl (Cys) and oxidation (Met) were specified as variable modifications. Cross-correlation scores of singly, doubly, and triply charged peptides >1.9, 2.2 and 3.75, respectively, were fixed for protein identification.

# DNA extraction, AS-PCR amplification, and cloning of secalin genes

Genomic DNA was extracted from dry seeds with a previously established cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson 1980). Genomic DNA was extracted from dry seeds by use of CTAB protocol (Murray and Thompson 1980). The  $\omega$ -secalin complete gene sequences (accessions FJ561478, FJ816038, FJ823443, X60294, AF000227, and FJ561465) from GenBank (www.ncbi.nlm.nih.gov/) were used for designing allelic specific PCR (AS-PCR) primers according to Wang et al. (2012a) named as forward Sec1 (5'-ATGAAGACCTTACTCATGCTTG-3') and reverse Sec2 (5'-CTATACCACTACTA CAAATGGT-3'). The AS-PCR amplifications were performed in a total volume of 50 µL containing 2.5 U LA Taq polymerase (TaKaRa), 60 ng of template DNA, 25 µL 2X GC buffer I (MgCl<sub>2</sub> plus), 0.4 mm dNTP, 0.5 µm of each primer, and double-distilled H<sub>2</sub>O. The PCR reaction was carried out in a S1000TM thermal cycler (Bio-Rad Laboratories Inc.) with the following program: an initial step of 94°C for 4 min; 34 cycles of 94°C for 45 s, 58°C for 1 min, and 72°C for 70 s; and a final step of 10 min at 72°C. The PCR products were analysed by agarose electrophoresis with 1% gel in Tris-acetic acid-EDTA buffer. The PCR fragments with expected sizes were purified from the gel using the Gel Extraction Kit (Omega Bio-Tek Inc., Norcross, GA, USA). The purified products were then ligated into pGEM-T Easy vector (Tiangen, Beijing, China) and transformed into cells of Escherichia coli TOP 10. The DNA sequencing from three clones of each PCR fragment was carried out by the Beijing Genomic Institute.

# Identification of 1BL.1RS translocation by PCR-based markers

Previously reported locus-specific primers (Chai *et al.* 2006) were used in the identification of the 1BL.1RS translocation. PCR amplifications were performed in a total volume of  $20 \,\mu\text{L}$  containing 1 U LA Taq polymerase (TaKaRa), 24 ng of template DNA,  $10 \,\mu\text{L} 2X \,\text{GC}$  buffer I (MgCl<sub>2</sub> plus),  $0.16 \,\text{mm} \,\text{dNTP}$ ,  $0.2 \,\mu\text{m}$  of each primer, and double-distilled H<sub>2</sub>O.

# RP-HPLC

The RP-HPLC analysis was performed on an Agilent 1100 instrument with reversed-phased column (ZORBAX 300SB-C18 Stable Band Analytical 4.6 by 250 mm, 5- $\mu$ m) (Agilent Technologies, Santa Clara, CA, USA). The major analytical parameters were set as 60°C for column temperature, 1.00 mL/min for flow rate, 20  $\mu$ L for sample volume, and eluting gradient and concentrations of ACN with 0.06% TFA gradually increasing from 21% to 47% (v/v) in 55 min. Column washing time between two adjacent samples was 15 min. The proteins were detected by measuring UV at 210 nm.

# RP-UPLC

The RP-UPLC was performed according to Yu *et al.* (2013*a*, 2013*b*) with some modifications on an Acquity UPLC<sup>TM</sup> (Waters Corporation) with a Waters 300SB C18 column ( $1.7 \mu$ m). Four solutions of elution buffers were used: solution A (ultra-pure water with 0.06% TFA), solution B (ACN with 0.06% TFA), solution C (ultra-pure water), and solution D (methanol). The column was balanced by increasing the concentration of solution B from 21% to 47% within 45 min. The ratios of solution A to B for weak washing and strong washing for needles were 79:21% and 53:47%, respectively. Sample washing was conducted with solution A from 95% to 5% and solution B from 5% to 95% in 5 min. Final washing was completed with solution C from 90% to 10%, and solution D from 10% to 90% three times within 30 min. At the end, 10 µL of solution D was injected into the column.

## Results

## Identification of 1BL.1RS translocations by A-PAGE

Gliadins, which are traditionally classified into three groups  $(\alpha/\beta-, \gamma-, \text{ and } \omega-\text{gliadins})$  on the basis of their electrophoretic

mobilities on A-PAGE gel (Woychik *et al.* 1961), were extracted from wheat cultivars by A-PAGE (Fig. 1). The omega secalins are encoded by *Sec-1* locus located on the short arm of chromosome IRS of rye (Shewry *et al.* 1984; Clarke *et al.* 1996). According to Sozinov *et al.* (1987), the gliadin Gli-1B3 area in the  $\omega$ -region can be used as 1BL.1RS translocation marker loci, in which the protein bands marked as *a*, *b*, and *c* are most likely to be omega secalins from rye (Fig. 1). Based on the A-PAGE results, 50 cultivars were found to have the Gli-1B3 site, probably containing the 1BL.1RS translocation, while 25 cultivars had no Gli-1B3 (see Supplementary Materials, Table 1).

# Characterisation of omega secalins from the 1BL.1RS translocation by LC-MS/MS

In order to confirm whether the three gliadin bands on the A-PAGE gels indicated in Fig. 1 belong to omega secalins from 1RS of rye, each band was excised from gels and purified. After digesting with trypsin, three proteins named as a, b, and c were further identified by LC-MS/MS and the results are shown in Table 1. According to the molecular weight and cover percentage of the peptide (Table 1 and Supplementary Materials Fig. 1), bands a and c were identified as omega secalins, whereas band b was proved to be wheat gliadin. The A-PAGE analysis also found that some cultivars such as Zhongyou 9507 contained band b (Fig. 1). As shown in Table 1, protein band a was well matched with the omega secalin ACQ83636.1, and two characteristic peptides (R.PFGQQPEQIISQR.P and R.QLNPS EQELQSPQQPVPK.E) were identified, which located at the positions 20-39 and 256-270, respectively. For band c, one characteristic peptide (R.QLNPSEQELQSPQQPVPK.E) well matched with omega secalin ACN96898.1 and located at the positions 20-39 (Table 1 and Supplementary Materials Fig. 1).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30



**Fig. 1.** Gliadin separation and 1BL.1RS translocation identification of 30 wheat cultivars by A-PAGE. The putative omega secalin bands *a*, *b*, and *c* translocated from rye are indicated. 1, Jing 411; 2, 05zhong 37; 3, Aikang 58; 4, Fu 98-46; 5, Hemai 0521; 6, Hongzhan 6816; 7, Huapei 212; 8, Huaimai 0566; 9, Jingjiumai 10; 10, Liuhu 98; 11, Yumai 47; 12, Hemai 026; 13, Meng 6112; 14, Luo 6112; 15, Meng 0318; 16, Yumai 34; 17, Zhoumai 11; 18, Zhoumai 12; 19, Heyou 1; 20, Jinmai 45; 21, Zhongyu 5; 22, Luofulin 10; 23, Luofulin 13; 24, Shanqianmai; 25, Aimengniu II; 26, Aimengniu IV; 27, Lumai 8; 28, Lumai 11; 29, Lumai 15; 30, Zhongyu 9507.

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The omega secalin genes at the Sec-1 locus from 10 cultivars that were identified to contain bands a and c were amplified and cloned by AS-PCR, and 20 non-redundant  $\omega$ -secalin genes were amplified. The deduced amino acid sequences of six representative genes (TaSec-NX1, TaSec-NX12, TaSec-NX13 from Neixiang 188, and TaSec-JD1, TaSec-JD2, TaSec-JD3 from Jingdong 8) were compared with previously characterised omega secalin genes (Supplementary Materials Fig. 2), and typical structural features of omega secalin genes of rye were found in all of these genes, including a conserved signal peptide of 19 amino acid residues started with RQL, a short N-terminal region of 12 amino acids, several repetitive domains (PQQP), and a short C-terminal region ending with four valine amino acid residues (De Vita et al. 2012). The two characteristic peptides of omega secalin identified by LC-MS/MS were present in conserved positions of all cloned genes (Supplementary Materials Fig. 2). These results demonstrated that the gliadin bands a and c are omega secalins resulting from the translocation of rye 1RS and can be used as reliable protein markers for the identification of 1BL.1RS translocations.

# Identification of 1BL.1RS translocation by PCR-based markers

Molecular markers are considered reliable and effective tools to identify different genotypes and can be used in any period of plant development (Bagge *et al.* 2007). As expected, AS-PCR by primer pair  $\omega$ -sec-P1 and  $\omega$ -sec-P2 resulted in a single 1.1 kb fragment and 40 cultivars were confirmed to carry the 1BL.1RS translocation, whereas no fragments were amplified from the 36 non-translocation cultivars (Fig. 2). The results were consistent with those by A-PAGE and LC-MS/MS as well as previous report (Chai *et al.* 2006).

# Identification of 1BL.RS translocation by RP-HPLC

Previous reports showed that wheat gliadins can be clearly separated into three regions based on their surface hydrophobicity and elution order, corresponding to  $\omega$ -,  $\beta/\alpha$ -, and  $\gamma$ -gliadins (Lookhart and Bean 1995*a*, 1995*b*; Wang *et al.* 2012*b*). The RP-HPLC analysis (Fig. 3) showed that the sample separation time was ~55 min, and three gliadin areas, corresponding to  $\omega$ -gliadins (14–28 min),  $\alpha/\beta$ -gliadins

(28–40 min), and  $\gamma$ -gliadins (40–54 min) could be clearly identified. In particular, a few minor peaks were present at 25–27 min in the  $\omega$ -gliadin area in the cultivars carrying the 1BL.1RS translocation, such as Aikang 58, Jingdong 8, and Neixiang 188 (Fig. 3), which were  $\omega$ -secalins by comparison with the cultivars without the 1BL.1RS translocation. Of the 76 wheat cultivars, 40 contained the 1BL.1RS translocation, consistent with the results of A-PAGE, LC-MS/MS, and PCR.

# Fast identification of 1BL.RS translocation by RP-UPLC

All wheat cultivars identified by A-PAGE, PCR, and RP-HPLC were further subjected to RP-UPLC analysis. The results showed that fast, highly reproducible, and clear separation of seed gliadins could be achieved by gradually increasing the eluting gradient from 21% to 47% over 30 min at a flow rate of 0.5 mL/min and separation temperature of 55°C (Supplementary Materials Table 2 and Fig. 3). The sample separation could be completed in ~27 min, much faster than traditional RP-HPLC method (Fig. 3). In order to test the reproducibility of RP-UPLC separation, 12 consecutive runs were performed for the separation of gliadins from Neixiang 188 under the optimised conditions. The averages, standard deviations, and relative standard deviations (RSD%) of migration time, peak height, and peak area of eight representative peaks are listed in Supplementary Table 2. Results indicated that a high level of reproducibility of RP-UPLC separation was achieved under the optimised conditions, with RSD% values being <0.1 for migration time, <4.0 for peak height, and <5.0 for peak area.

Similar to the RP-HPLC pattern, three gliadin areas were also well separated by RP-UPLC, in which  $\omega$ -gliadins eluted at 10–15 min,  $\alpha/\beta$ -gliadins at 15–23 min, and  $\gamma$ -gliadins at 23–27 min. All cultivars that were identified to carry the 1BL.1RS translocation by A-PAGE, RP-HPLC, and PCR showed a few minor peaks at 12–13 min in the  $\omega$ -gliadin area, which corresponded to  $\omega$ -secalins, making it differentiable between the 1BL.1RS translocation and non-translocation cultivars (Fig. 4). As for the results of A-PAGE, RP-HPLC, and PCR, 40 bread wheat cultivars were identified to have the 1BL.1RS translocation, indicating that a higher percentage of 1BL.1RS translocation is present in the recently released bread wheat cultivars in China.



Fig. 2. PCR identification of wheat 1BL.1RS translocation. A specific amplification fragment with 1 kb for omega secalin genes from 1BL.1RS translocation was arrowed.



Fig. 3. Gliadin separation and 1BL.1RS translocation identification of eight wheat cultivars by RP-HPLC. The omega secalin peaks from the 1BL.1RS translocations are arrowed.



Fig. 4. Separation of seed gliadins and identification of 1BL.1RS translocations from 20 wheat cultivars by RP-UPLC with optimised conditions. The omega secalin peaks from 1BL.1RS translocations are arrowed.

# Discussion

The 1RS chromosome arm is one of the most intensively used rye chromosome segments as the form of a 1BL.1RS translocation in wheat breeding (Rabinovich 1998; Graybosch 2001). So far, numerous methods at the morphological, cell, protein, and molecular levels have been developed to identify this translocation. However, although each method has its own advantages, disadvantages are obviously present (Table 2). Thus,

l	Table 2.       Relative effici	encies of seven main gl	iadin analysis methods	for identifying wheat	t 1BL.1RS translocat	ion	
	A-PAGE	2-DE	LC-MS/MS	PCR	HPCE	RP-HPLC	RP-UPLC
Required sample amount Required sample purity	~40 µg (protein) Low	~150 µg (protein) High	~0.04 µg (protein) High	~10 ng (DNA) Medium	~1 µg (protein) Medium	~2.5 µg (protein) Medium	~0.5 µg (protein) Medium
Mass accuracy	Inaccurate	Inaccurate	Accurate	Accurate	Inaccurate	Unknown	Unknown
pI	Unknown	Known	Known	Unknown	Unknown	Unknown	Unknown
Time consumed for one sample testing	$\sim$ 5 $-8$ h	>24 h	$\sim 5-10 \min$	>10h	~50 min	~60 min	$\sim 25 \text{ min}$
Cost of equipment	Low	Medium	Much high	Medium	High	High	High
Analysis cost	Low	High	High	Medium	Low	High	Low
Automation level	Low	Low	High	Medium	High	High	High
Experience required	Medium	Considerable	Less	Medium	Less	Less	Less
Safety	High toxicity	High toxicity	Safe	Toxicity	Safe	Safe	Safe
False positives	No	Yes	No	Yes	No	No	No
Accuracy level	++	++++	++++	++	+	‡	+

Since electrophoretic methods are simple and easy to perform in most laboratories, they are suitable for large-scale and high-throughput 1BL.1RS translocation screening in breeding programs. Thus, A-PAGE has become a widely used method for gliadin analysis, by which 1BL.1RS translocation identification can be achieved (Fig. 1). However, its disadvantages are also obvious, including use of toxic reagents and a slow, labour-intensive procedure with relatively poor resolution and reproducibility. Use of 2-DE and LC-MS/MS can obtain a much high reliability for protein identification, but the sophisticated operation procedures and expensive equipment as well as high analysis costs limited their wide application.

Molecular markers have shown to be an effective tool for identifying desirable genes and genotypes in plant breeding programs (Bagge et al. 2007). The current study (Fig. 2) and a previous report (Chai et al. 2006) demonstrated that PCR-based markers readily differentiate the cultivars carrying the 1BL.1RS translocation, with a throughput and accuracy similar to A-PAGE. Its main drawbacks include that it is highly timeconsuming and costly for DNA extraction, PCR reaction, and electrophoretic separation of the amplification products, and false positives are possible.

Various capillary electrophoresis and liquid chromatography methods can realise automatic protein separation with high resolution and reproducibility (Yan et al. 2003; Dong et al. 2009; Gao et al. 2010). For example, both RP-HPLC and HPCE can separate and identify wheat storage proteins with the features of easy use and flexible operation along with stable machine performance (Bietz 1983; Yan et al. 2003). However, RP-HPLC generally needs a long separation time and large amount of analytical reagents. Although HPCE can differentiate between 1AL.1RS and 1BL.1RS translocations (Lookhart et al. 1996), it is difficult to distinguish 1BL.1RS translocation and non-translocation cultivars.

Our results showed that, although both RP-HPLC and RP-UPLC can clearly identify the 1BL.1RS translocation in wheat cultivars based on a few specific peaks in the  $\omega$ -gliadin region (Figs 3 and 4), RP-UPLC displayed a much faster separation and a significantly lower sample and analytical reagent requirement (Table 2). Therefore, RP-UPLC represents a more efficient way for identifying the wheat 1BL.1RS translocation with lower costs, and it is expected to become a powerful tool for wheat cultivar differentiation and germplasm screening.

In summary, for the identification of the 1BL.1RS translocation in wheat improvement programs, fast gliadin separation with low costs, high throughput, and reliability are particularly important. For rapid identification and largescale screening of the 1BL.1RS translocation in wheat quality breeding, RP-UPLC is expected to be an appropriate and alternative method.

#### Acknowledgements

This research was financially supported in part by grants from the National Natural Science Foundation of China (31271703, 31101145), the Chinese Ministry of Science and Technology (2009CB118303) and the National Key Project for Transgenic Crops of China (2011ZX08009-003-004).

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