Agnieszka Niedziela*, Piotr Bednarek

Department of Plant Physiology and Biochemistry, Plant Breeding and Acclimatization
Institute — National Research Institute, Radzików, 05-870 Błonie, Poland;
*Corresponding author e-mail: a.niedziela@ihar.edu.pl

CHARACTERIZATION OF DART SEQUENCES REFLECTING GENOMICREGIONS INVOLVED IN ALUMINUM TOLERANCE IN TRITICALE (X *TRITICOSECALE* WITTMACK)

ABSTRACT

Aluminum toxicity is the major growth-limiting factor for crop cultivation on acid soils. Tolerance mechanisms for Al stress in triticale have not been systematically investigated so far. It is presumed, that in the case of this species they may be a function of the interaction between wheat and rye genes. In this study the sequences of forty-six Diversity Arrays Technology markers associated with aluminum tolerance in triticale and under selection pressure were blasted against BLAST database for the identification of possible functions of the respective genome regions in Al-stress response. The analysis has showed sequences similarity to the domains involved in signaling, disease response and DNA repair mechanisms.

Key words: aluminum tolerance, triticale

INTRODUCTION

Aluminum (Al) tolerance is an important trait that allows crop production on acidic soils occupying over 50% of the world's arable land. The Al released from soil minerals under acid conditions (pH<5) occurs as $Al(OH)^{2+}$, $Al(OH)_2^{+}$ and $[Al(H_2O)_6]^{3+}$ ions, which can be readily uptaken by the roots (Foy 1992). For most agriculturally essential plants, Al ions rapidly inhibit root growth, damage root systems, and cause a significant reduction in crop yields. Therefore, the identification of genes involved in Al-tolerance is of supreme importance for plant production in the world. Several mechanisms related to Al-tolerance are known, but the most mattering one relays on the ability of organic acids to chelate Al^{3+} ions via the formation of low molecular weight complexes (Kochian 1995). There are two gene families responsible for organic acid exudation in cereals. The ALMT (aluminum-activated malate transporter) family is accountable for malate, whereas MATE (multidrug and toxin efflux) one for citrate exudation. In triticale, the most basic gene coding for ALMT is located on the 7R chromosome and explains up to 36%

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of the phenotypic variance (Niedziela *et al.* 2014). The other genes were found on chromosomes 3R (Ma *et al.* 2000; Budzianowski and Woś 2004; Niedziela *et al.* 2012), 4R and 6R (Niedziela *et al.* 2012) but their function in triticale genome was not recognized. However, in rye, the *Alt2* locus located on the chromosome 3R encodes a putative STOP1 transcription factor that may regulate the expression of genes coding for both organic acids (Silva-Navas *et al.* 2011). Moreover, Gallego and Benito (1997) reported linkages of the *Alt1* locus assigned to 6R rye chromosome with the aconitase-1 (Aco1), nicotinamide adenine dinucleotide dehydrogenase-2 (Ndh2), esterase-6 (Est6) and esterase-8 (Est8) genes. Nevertheless, Al-resistance is a complex phenomenon resulting from several biochemical mechanisms including tricarboxylic acid (TCA) cycle, anti-oxidant, pathogen defense, signal transduction and general stress-responsive pathway (Milla *et al.* 2002, Guo *et al.* 2007).

Studies that may support the identification of genes conferring Al-tolerance in triticale could be conducted using modern high-throughput DNA marker systems such as, i.e., Diversity Arrays Technology (DArT) technique. DArT is a hybridization-based genotyping technology that allows for the evaluation of several hundred polymorphic loci spread over a genome, without any previous sequence knowledge (Wenzl *et al.* 2004). DArT involves the use of methylation-sensitive restriction enzymes, usually *Pst*I, in the genome complexity reduction stage, thereby allowing the evaluation of markers, predominantly originating from the hypomethylated, low-copy, and gene-rich regions (Jaccoud *et al.* 2001). The marker sequences (if available) provide the info on their putative homology to functional genes and for candidate gene identification (Petroli *et al.* 2012; Gawroński *et al.* 2016).

Previously, we have applied the DArT markers for the genome-wide association mapping of Al-tolerance in triticale (Niedziela *et al.* 2012) identifying 52 and 47 ones associated with Al-tolerance and under selection pressure, respectively. The markers mapped to the 3R, 4R, 6R, and 7R chromosomes. Based on the chromosomal location we suggested that the Al-activated malate transporter (ALMT) gene, present on chromosome 7R, may contribute to the trait (Niedziela *et al.* 2014). However, we did not focus on the evaluation of homology of the DNA marker sequences and those available in DNA databases. Thus, we did not check for putative genes associated with Al tolerance.

The study primary aims to evaluate homology of the Al-associated (AS) marker sequences and those under positive selection (SP) and balancing pressure (BP) to the sequences deposited in databases following the identification of the putative genes involved in Al-tolerance in triticale and they role in Al stress response.

MATERIALS AND METHODS

The DNA sequences of DArT markers associated with aluminium tolerance in triticale and under selection pressure were kindly provided by A. Kilian (Diversity Arrays Technology P/L, Canberra, Australia) and M. Tyrka (Rzeszów University of Technology, Poland). Association mapping was conducted and described in our previous study (Niedziela *et al.* 2012) using 232 triticale breeding forms originating from Experimental Station (Małyszyn). Plant materials were phenotyping according to the standard protocol described by A. Anioł (1984). Briefly, sterilized and germinated seeds were sown on polyethylene nets floated in a tray filled with basic medium (Anioł, 1984).

Three days old seedlings were transferred for 24 h onto the same medium containing Al^{3+} ions (16 ppm) in the form of AlCl$_3$. Next, the plants were washed in water and then placed again in the basic medium for 48 h. To evaluate the Al response, the length of root regrowth in mm was measured. The DNAs were extracted from leaves of 7-day old seedlings and genotyped with Diversity Arrays Technology (DArT). Association mapping was completed in TASSEL (Bradbury *et al.* 2007) using General (GLM) and Multiple (MLM) Linear Models. Additionally, Statistical Machine Learning (SML) approach was also employed (Bedo *et al.* 2008). Furthermore, markers reflecting genomic regions under putative positive (PS) and balancing selection (BS) were identified (Niedziela *et al.* 2012). DArT marker sequences were check for their redundancy (Niedziela *et al.* 2015) using CLC Main Workbench software version 6.0 (http://www.clcbio.com/) and the UPGMA approach for clustering.

In this study, non-redundant sequences of DArT markers associated with aluminum tolerance in triticale and under selection pressure were blasted using BLASTn against a GeneBank of The National Centre for Biotechnology Information (NCBI) database. Classification of the query sequences was based on: (1) Identity (I-% of the similarity between the query and subject sequences over the length of the coverage area); (2) Query Cover (QC-% of the query sequence that overlaps the subject sequence) and 3) E-value (homology probability value) criteria. The threshold for reporting matches (E) equalled to 10.0E-10. The taxonomic category selected was the *Poaceae* family.

RESULTS

Out of 52 DArT markers associated with Al-tolerance, sequences of 10 were redundant whereas another 10 were unavailable (Niedziela *et al.* 2015). Thus, we ended with 32 marker sequences. Moreover, 8 markers under positive and 18 once under balance selection pressure were included in the analysis (Niedziela *et al.* 2012). All 58 marker sequences ranging from 106 to 915 bp were selected for the search of homology to the DNA sequences deposited in DNA databases. Following BLASTn analysis 49 of highly significant hits (*E-value > 10E-10, Table1) for DArT sequences available in wheat (*Triticum aestivum* and *Triticum turgidum*), barley (*Hordeum vulgare* subsp. *vulgare*), Tausch's goatgrass (*Aegilops tauschii* subsp. *tauschii*) and purple false brome (*Brachypodium distachyon*) databases were identified. In detail, eight sequences coding acid beta-fructofuranosidase precursor, ER body-like protein, cyclin-P4-1-like, syntaxin-32, HGV2-like protein, avenin-like a precursor and pdil5-1 gene as well as the sequence of the miniature inverted-repeat transposable element (MITE) Tourist-5 were associated with Al-tolerance. Four sequences under positive selection indicated the involvement of disease resistance protein RGA2-like, lysM domain-containing GPI-anchored protein 1 and prolamine gene in the expression of the trait. Finally, ten disease resistance proteins RPS2-like, RPP13-like and RGA1, ferredoxin-NADP(H) oxidoreductase, NBS-LRR-like protein gene, acid beta-fructofuranosidase precursor, putative receptor-like protein kinase At3g47110, wall-associated receptor kinase 4-like, DNA repair protein Rad50 gene and putative avenin-like precursor matched DNA sequences under balancing selection pressure (Table 1). Among them, twenty-two matched genes of a known or putative function with query cover (QC %) reached up to 45% for half of them and E-value from 8.0E-11 to 0.0 (Table 1).

Table 1

DArT markers associated (AS) with aluminum tolerance or under positive (PS) or balance (BS) selection with significant similarity (BLASTn) to known sequences of genes/proteins. Only scores with the highest E-value were showed.

Chr	Marker name	Size bp	Mached species	Mached gene/sequence	QC [%]	E-value	I [%]	Accession number
				Associated markers (AS)				
4R	rPt-410768	579	*Aegilops tauschii*	PREDICTED: uncharacterized LOC109775811	74	4.00E-151	93	XM_020334511.1
4R	rPt-507784	621	*Triticum aestivum*	acid beta-fructofuranosidase precursor (inv1 gene)	9	2.00E-16	92	AJ635225.1
4R	rPt-508577	411	*Triticum aestivum*	MITE Tourist-5	63	2.00E-95	90	FJ345691.1
4R	rPt-505674	691	*Aegilops tauschii*	PREDICTED: membrane protein of ER body-like protein	44	7.00E-86	95	XM_020344060.1
4R	rPt-509188	732	*Aegilops tauschii*	PREDICTED: cyclin-P4-1-like	32	7.00E-80	89	XM_020322112.1
4R	rPt-402237	397	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	100	2.00E-65	76	HG670306.1
4R	rPt-402563	336	*Aegilops tauschii*	PREDICTED: uncharacterized, transcript variant X7	61	2.00E-81	93	XR_002229381.1
6R	rPt-399834	274	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	88	4.00E-33	74	HG670306.1
6R	rPt-505347	530	*Aegilops tauschii*	PREDICTED: syntaxin-32	23	2.00E-33	88	XM_020298320.1
6R	rPt-509167	459	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	49	1.00E-42	80	HG670306.1
6R	rPt-508379	684	*Triticum aestivum*	pdil5-1 gene for putative PDI-like protein	13	8.00E-11	91	FN555311.1
6R	rPt-506198	695	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	51	3.00E-53	84	HG670306.1
6R	rPt-505870	647	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	24	4.00E-12	82	HG670306.1
6R	rPt-401083	526	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	92	1.00E-29	79	HG670306.1
6R	rPt-402015	710	*Brachypodium distachyon*	PREDICTED: uncharacterized LOC100830473	59	7.00E-42	74	XM_010240565.1
7R	rPt-508078	598	*Aegilops tauschii*	PREDICTED: protein HGV2-like	50	5.00E-36	72	XM_020295220.1
7R	rPt-505154	547	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	27	4.00E-11	83	HG670306.1
7R	rPt-401828	598	*Hordeum vulgare*	mRNA for predicted protein, partial cds, clone: NIASHv2127M20	32	9.00E-33	77	AK371193.1
7R	rPt-399570	203	*Triticum aestivum*	mRNA for putative avenin-like a precursor (avnla gene)	23	8.00E-15	100	AM087940.1
7R	rPt-399325	106	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	98	2.00E-11	80	HG670306.1

Continued

Table 1

Chr	Marker name	Size bp	Matched species	Matched gene/sequence	QC [%]	E-value	I [%]	Accession number
				Markers under positive selection (PS)				
3R	rPt-508975	837	*Aegilops tauschii*	PREDICTED: disease resistance protein RGA2-like	34	4.00E-52	85	XM_020339618.1
3R	rPt-400318	505	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	91	2.00E-77	84	HG670306.1
3R	rPt-508819	542	*Hordeum vulgare*	mRNA for predicted protein, complete cds, clone: NIASH-v2100A23	98	0.00	95	AK369867.1
3R	rPt-402334	449	*Hordeum vulgare*	mRNA for predicted protein, complete cds, clone: NIASH-v2090H12	99	5.00E-149	86	AK369399.1
4R	rPt-505775	536	*Aegilops tauschii*	prolamin gene locus	20	4.00E-31	92	JX295577.2
4R	rPt-400317	225	*Aegilops tauschii*	prolamin gene locus	46	7.00E-29	92	JX295577.2
6R	rPt-401893	362	*Aegilops tauschii*	PREDICTED: lysM domain-containing GPI-anchored protein 1	61	1.00E-54	89	XM_020343358.1
7R	rPt-400793	370	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	100	7.00E-152	93	HG670306.1
				Markers under balance selection (BS)				
4R	rPt-506527	656	*Triticum turgidum*	DNA repair protein Rad50 gene	46	4.00E-88	85	EU159424.1
4R	rPt-508454	363	*Aegilops tauschii*	PREDICTED: disease resistance protein RPS2-like	45	8.00E-44	84	XM_020321759.1
4R	rPt-506540	636	*Aegilops tauschii*	PREDICTED: putative disease resistance RPP13-like protein 3	100	0.00	90	XM_020304320.1
4R	rPt-507894	728	*Aegilops tauschii*	PREDICTED: uncharacterized LOC109734896	46	2.00E-29	67	XM_020294081.1
4R	rPt-507403	527	*Triticum aestivum*	mRNA for ferredoxin-NADP(H) oxidoreductase (fnr gene)	15	1.00E-29	96	AJ457980.1
4R	rPt-506534	547	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	65	4.00E-75	80	HG670306.1
6R	rPt-401470	333	*Aegilops tauschii*	PREDICTED: putative disease resistance protein RGA1	68	2.00E-19	73	XM_020330139.1
6R	rPt-509728	722	*Hordeum vulgare*	NBS-LRR-like protein gene	99	0.00	86	AF414177.1

Continued

Table 1

Chr	Marker name	Size bp	Matched species	Matched gene/sequence	QC [%]	E-value	I [%]	Accession number
				Markers under balance selection (BS) - continued				
6R	rPt-411086	521	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	92	1.00E-29	79	HG670306.1
6R	rPt-505673	714	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	62	8.00E-35	82	HG670306.1
6R	rPt-509333	668	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	34	6.00E-11	81	HG670306.1
6R	rPt-401554	542	*Triticum aestivum*	mRNA for acid beta-fructofuranosidase precursor (inv1 gene)	11	1.00E-17	94	AJ635225.1
7R	rPt-401221	467	*Aegilops tauschii*	PREDICTED: putative receptor-like protein kinase At3g47110	91	3.00E-127	82	XM_020301520.1
7R	rPt-508868	757	*Aegilops tauschii*	PREDICTED: wall-associated receptor kinase 4-like	70	0.00	91	XM_020332070.1
7R	rPt-506250	441	*Triticum aestivum*	mRNA for putative avenin-like a precursor (avnla gene)	17	7.00E-14	100	AM087940.1
7R	rPt-401363	484	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	84	1.00E-93	78	HG670306.1
7R	rPt-509288	458	*Triticum aestivum*	chromosome 3B, genomic scaffold, cultivar Chinese Spring	50	5.00E-54	93	HG670306.1
7R	rPt-402262	554	*Aegilops tauschii*	PREDICTED: uncharacterized LOC109754523	99	0.00	96	XM_020313430.1

Description:
Chr–chromosome; QC–Query cover (% of the query sequence that overlaps the subject sequence), I– identity (% of the similarity between the query and subject sequences over the length of the coverage area); E-value– homology probability value; Accession number –access number in GenBank of homolog sequence.

E-value scale:
E-value < 10E-100 identical sequences; 10E-100 < E-value < 10E-50 almost identical sequences, a long stretch of the query protein is matched to the database; 10E-50 < E-value < 10E-10 closely related sequences, could be a domain match or similar; 10E-10 < E-value < 1 could be a true homologue but it is a grey area; E-value > 1 proteins are most likely not related.

DISCUSSION

Plants have developed several distinct strategies to cope with Al toxicity. External and internal detoxification mechanisms that act synergistically to protect plants are proposed and studied intensively for about 30 years. During the last decade, a variety of genes expressed upon Al exposure have been recognized in a range of plant species, including crops (Ryan *et al.* 2009; Ma *et al.* 2014; Kochian *et al.* 2015; Nguyen *et al.* 2001). However, only limited efforts have been made to the identification of Al responsive genes in triticale (Niedziela *et al.* 2014).

For years, DArT markers were used as anonymous genomic markers for genetic diversity analysis, linkage map construction, QTL identification and association mapping (Wenzl *et al.* 2004; Bolibok-Brągoszewska *et al.* 2009; Tyrka *et al.* 2018). Since the DArT marker sequences become publicly available (with some exceptions) they may provide a very useful tool for the identification of candidate genes for traits of interest. In this study DArT markers classified as being associated with Al-tolerance and under selection pressure (Niedziela *et al.* 2012) were investigated for the identification of the putative genes involved in Al-tolerance in triticale. One of the highest homology probability values (E-value) were obtained for eight DArT sequences similarities to the domains implicated in signalling processes and disease response in plants. Among them a receptor-like protein kinase (3.00E-127), wall-associated receptor kinase (0.00), RGA1(2.0E-19), RPS2-like (8.00E-44), RPP13-like protein 3 (0.00) and NBS-LRR-like (0.00) share homology with markers being under balance selection pressure, and the RGA2-like (4.00E-52) as well as lysM and domain-containing GPI-anchored protein 1 (1.00E-54) with markers being under positive selection pressure. Selection pressure affects an organism ability to survive in the given environment (Grenier *et al.* 2016). Positive selection is related to the tendency of beneficial traits to increase in prevalence in the population (Roth and Liberles 2006), whereas balancing selection (Delph and Kelly 2014) occurs when multiple phenotypes or alleles are actively maintained in the population. The triticale lines used in the association study were regularly tested on media supplemented with aluminium and only lines with desired phenotypes were selected which led to an increase in the frequency of favoured alleles. Our study suggests that the selection process favoured sequences coding disease resistance (R) genes. If expressed, the products could serve as sensors/receptors of aluminium stress. Their role in metal stress detection likely arises from the fact that both, pathogens as well as abiotic factors (e.g., aluminium) evoke oxidative stress by generating reactive oxygen species (ROS) (Tameling and Joosten 2007; Mandal *et al.* 2013). The R genes with TIR (N-terminal toll/interleukin) - NBS-LRR (nucleotide-binding site-leucine-rich repeat) domain was activated in *Medicago truncatula* treated with mercury (Zhou *et al.* 2012), in *Raphanus sativus* treated with chromium (Liu *et al.* 2015), and in *Glycine max* under aluminium stress (Zeng *et al.* 2012). It was also supposed (Fan *et al.* 2016), that disease resistance proteins with NBSs domains required for ATP and GTP binding may be associated with membrane-bound ion channel ATPases, that import many of the metabolites necessary for cell metabolism and export toxins which

hinders cellular processes (Marone *et al.* 2013). As we have identified markers with sequence homology to kinases, one may speculate that R-genes are mediated by these enzymes (Goff and Ramonell 2007; Kanneganti and Gupta 2008). Another marker under balance selection pressure with high sequence similarity (4.00E-88) to the sequence of the DNA repair protein Rad50 gene belongs to MRE11-RAD50-NBS1 (MRN) complex involved in detection of DNA damage and activation of cell-cycle checkpoints and double-strand breaks (DSBs) repair via recombination (Nezames *et al.* 2012). It was described that Al induces a series of cellular damages in the growing root tip adversely affecting cell division and nucleolus (Zhang *et al.* 2014). The presence of DArT marker with sequence homology to the Rad50 gene implicates the vital role of MRN complex in the detection of DNA damage in triticale affected by Al. The researches with the loss-of-function rad17-1 mutant in *Arabidopsis thaliana* show a mild increase in sensitivity to Al, likely because of failure to initiate repair of DNA damage that occurs with Al treatment (Nezames *et al.* 2012).

In our study markers rPt-509188, rPt-505674, and rPt-508577 associated with Al-tolerance on 4R chromosome show sequence homology reaching E-value over 7.00E-80 to the sequences of cyclin-P4-1-like, a membrane protein of ER body-like (MEB) protein and tourist-like miniature inverted-repeat transposable element (MITE Tourist-5), respectively. Interestingly, the markers were detected exclusively in Al-tolerant genotypes (not shown). The cyclin-P4-1-like protein is involved in regulation of cyclin-dependent protein serine/threonine kinase activity (CDKs) and cell division via phosphorylation of critical substrates (such as the retinoblastoma protein, transcription factors, nuclear laminar proteins, and histones) (Torres Acosta *et al.* 2004). MEB1 and MEB2 proteins found in *Arabidopsis thaliana* belong to the vacuolar iron transporter (VIT) family characterized by the presence of a DUF125 sequence, which underlies their ability to transport metal ion (Yamada *et al.* 2013). Those proteins are functional homologs of the yeast iron transporter CCC and can partially reduce the iron toxicity in the yeast ccc1 mutant (Yamada *et al.* 2013). Moreover, in rice OsVIT1 and OsVIT2 transport iron and zinc, and are responsible for the accumulation of these metals (Zhang *et al.* 2012). However, the relationship between MEB proteins and Al transport has not been documented so far. The presence of marker related to the transposable element may be directly linked to Al-tolerance genes. It was documented that the presence of transposable elements in promoter region influenced the expression of MATE gene in *Sorghum bicolor* (Magalhaes *et al.* 2007) and ALMT gene in *Triticum aestivum* (Sasaki *et al.* 2006; Tovkach *et al.* 2013). A similar function cannot also be excluded in case of triticale.

It is interesting that as many as four markers in our study showed sequence homology to the genes coding for the seed storage proteins. Analysis of seed storage gene (coding for prolamins and avenins) location in triticale genome suggests that they map to several chromosomes including the 7R, the one that codes for the ALMT gene. Unfortunately, the data concerning the linkage of the two genes is not available. However, we cannot exclude that it exists. If so, this might be the reason for our result indicating an association of seed storage proteins and Al tolerance.

CONCLUSIONS

In this study twenty-two annotations to the known genes/proteins were found based on the DNA sequences of DArT markers associated with Al-tolerance and being under selection pressure. Considering the length of the DArT marker sequences (usually 400-800 nucleotides) and their localization in gene-rich regions determined by methylation sensitive *Pst*I enzyme used for analysis as well as highly significance results of BLAST search we may suspect that our data provides reliable information on possible functions of the respective genome regions in Al-stress response. Nerveless, exact knowledge of triticale response to aluminum stress still requires further studies.

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