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Chromosomal localization of selected SCARs converted from new RAPD, ISSR and R-ISSR markers linked to rye (*Secale cereale* L.) tolerance to nutrient deprivation stress identified using bulk segregant analysis

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Abstract

Background: An adaptive mechanism in plant roots is initiated in the event of nitrogen and potassium deficiency, and it facilitates the active uptake of these elements in order to ensure plant growth and survival in stress conditions. Signaling and transduction of signals in response to changing nitrogen and potassium concentrations is a complex process, affected by interactions between various gene expression products, and often subjected to modifications.
Results: In order to identify genotypic differences between phenotypes of two populations of recombinant inbred rye lines (153/79-1 x Ot1-3 and Ot0-6 x Ot1-3) in response to nutrition stress caused by nitrogen and potassium deficiency at the seedling stage, bulk segregant analysis was utilized. Identification of genotypic differences between and within pooled DNA samples involved 424 RAPD, 120 ISSR primers and 50 combinations of R-ISSR. Identified markers were sequenced and converted to SCAR, attributing to them unique ESTs annotations, and chromosomal ones to selected localizations. Significant relationships with the examined trait were described for nine and eight RAPD markers, four and five ISSR, one and three R-ISSR markers for population 153/79-1 x Ot1-3 and Ot0-6 x Ot1-3, respectively. Sequences identified for the rye genome were characterized by a uniqueness and a similarity to the sequence of aquaporin PIP1, a gene encoding protein related to the function of the transcription factor in plant response to iron deficiency and the putative ethylene-responsive transcription factor, cytosolic acetyl-CoA carboxylase, HvHKT1 transporter, as well as HCBT proteins.
Conclusion: Identified molecular markers differentiating rye genotypes of extreme response of root system on nitrogen and potassium deficiency play a significant role in systemic plant response to stress, including stress caused by nitrogen and potassium deficiency. They may constitute a system facilitating selection, and together with the material they are described in, they may be a starting point for research on mechanisms of sensing and transduction of signal across the plant.

Keywords: molecular markers, nutrient deficiencies, orthologs, response, RILs, rye, TIGR, TOGA.

INTRODUCTION

Plant response to nutrition stress caused by deficiency of both nitrogen (N) and potassium (K) in the soil is different and depends on genotype (Tuberosa and Salvi, 2007). As a response to stress, some genotypes increase root to shoot ratio, modify root morphology (Zhang et al. 2007), re-mobilize N resources (Andrews et al. 2006), and allocate assimilates from the leaves to the roots (Hermans et al. 2006). Other genotypes inhibit root growth and elongation. In the first case, the accepted strategy

enables better soil penetration by the roots increasing the chance of finding nutrients and uptake from deeper soil layers, while in the second case it allows the plant to survive (Bloom et al. 1985; Van der Ploeg et al. 1999).

In response to potassium deficiency, plant roots activate transporters and channels (*i.a.* HAK5 and AKT1 in *A. thaliana*), such as those involved in changes in primary root growth, and modify the formation of lateral- and hair-root elongation (López-Bucio et al. 2003, Schachtman and Shin, 2007; Jung et al. 2009; Kim et al. 2012). Generally, plants under K-deprived conditions exhibit poorly developed roots. Root-hair elongation is stimulated, but lateral root elongation is inhibited. Generally, plants under K-deprived conditions exhibit poorly developed roots. Root-hair elongation is stimulated, but lateral root elongation is inhibited (Jung et al. 2009; Tsay et al. 2011).

Adaptive strategies of plants with respect to nitrogen and potassium deficiency may be considered on molecular, morphological and ecological levels. The first are focused on recognition of the pathways of registration, processing and transmission of external and internal signals informing the plant of the state of nutrient availability (N and K). The second, based on analysis of morphological reactions, enable selection of desirable genotypes as genes donors for new varieties provided that the desirable genotype, which tolerates nutrition stress conditions, is actively searching for nutrients via changes in *e.g.* root morphology or active allocation of assimilates from shoots to roots (Andrews et al. 2006; Smolik, 2013a). The third enable an understanding of the fact that over-fertilization of crops (*e.g.* with nitrogen) does not contribute to increased crop growth, but only increases the cost of production, as well as eutrophication range (Raun and Johnson, 1999, Liu et al. 2008).

Despite the fact that NO_3^- constitutes a nutrient source, it is also a signaling molecule. NO_3^- induces expression *e.g.* of genes for nitrate uptake (NRT) and assimilation (NIA, NIR). The first element of the signaling pathway for NO_3^- is the dual-affinity nitrate transporter CHL1 (NRT1.1) - a sensor and concurrently a transceptor, the second is NRT2.1. In the event of NO_3^- deficiency, CHL1 is subject to phosphorylation, as it is a high-affinity transporter, and the changes in the expression of genes engaged in nitrate response are small, while NRT2.1 is subject to upregulation. With an excess of NO_3, CHL1 (in T101) is dephosphorylated, functions as a low-affinity transporter, and primary nitrate response genes are significantly upregulated (Tsay et al. 2011). It should be added that the process of the phosphorylation or possible dephosphorylation of CHL1 also involves other transporter genes, such as CIPK23 (calcineurin B-like interaction protein kinase 23) and CIPK8, expressed at a similar time, but independently of external NO_3^- ion concentrations (low- or high-affinity response, respectively) (Xu et al. 2006; Castaings et al. 2009; Tsay et al. 2011).

With various concentrations of NO_3^- ions in the cell, their interaction with transcription factors is noted, and this affects the expression of various genes, leading to changes in plant morphology, including its root architecture (Castaings et al. 2009).

Such regulators include nitrate-induced gene encoding transcription factor NLP7. It is a positive regulator which, in the case of high internal NO_3^- concentrations, presumably inhibits plant developmental adaptation to N-deficiency (Castaings et al. 2009). Another transcription factor is the *ANR1* gene (a MADS-box family member) participating in the signaling pathway of root elongation (Zhang and Forde, 1998; Zhang et al. 1999). Zhang et al. (1999) proved that stimulation of lateral root elongation appears to be attributable to a signaling effect from the NO_3^- ion. It was noted that the feedback mechanism is initiated with high concentrations both in the cells and in the surrounding roots, and this inhibits their elongation. However, its functioning has not yet been explained.

The mechanism is known for the inhibition, in the cell, of the expression in genes regulating uptake and assimilation of nitrogen (Cooper and Clarkson, 1989; Walch-Liu et al. 2006) by an accumulation of nitrogen metabolites, *i.e.* glutamine or glutamate (Vidmar et al. 2000). In high concentrations - LBD37 (lateral organ boundary domain), a transcription factor encoded by *LBD37* gene leads to the inhibition of the expression of nitrate-responsive genes, thus inhibiting NO_3^- uptake (Tsay et al. 2011). Similar regulation has been described for another transcription factor, CCA1 encoded with the *CCA1* gene (master clock control) (Gutiérrez et al. 2008). Also, the gene encoding miR393 is upregulated, significantly affecting expression (down regulate) of the auxin receptor -gene *AFB3* (auxin signaling F-box 3) through its transcript digestion. Thus, root growth is inhibited (Vidal et al. 2010; Shin, 2011; Schachtman, 2012).

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Despite the fact that the general mechanism of plant response to K deficiency is known, numerous elements regulating this response are still unclear. It was demonstrated that deficiency of potassium actively stimulates the element uptake of plant roots by activation of transport proteins and ion channels. Among many described factors, significant importance for this response has been attributed among other things to inwardly rectifying K^+ channel (AKT1) and HAK5 transporter (Schachtman and Shin, 2007; Jung et al. 2009). Shin and Schachtman (2004) found that low K^+ not only stimulates ROS (reactive oxygen species) production in roots, thereby inducing expression of *HAK5*, but also, as it was demonstrated by the authors, contributes to an increase in ethylene production. It was revealed that ethylene signaling acts upstream of ROS when plants are deprived of K^+ (Jung et al. 2009). According to a study by Nieves-Cordones et al. (2008), also root plasma membrane potential may be involved in transcription regulation of *HAK5*. Also CIPK23 protein kinase plays an important role in systemic response to deficiency of this element, which confirms the significant role of calcium in the potassium deficiency signaling pathway and its influence on activation of AKT1 potassium channels, as well as a range of hormones with significant part of not only above-mentioned ethylene but also auxins, jasmonic acid or ABA (Shin, 2011).

The availability of K^+ ions plays a significant role in the conditions of stress caused by N deficiency. It has been demonstrated that N metabolism depends on the presence of K^+ ions. Li et al. (2006), Xu et al. (2006) and Lee et al. (2007) observed that CIPK23 protein kinase forms a protein complex in response to low concentrations of potassium in soils. They noted that this protein activates the AKT1 channel via phosphorylation, and probably inactivates it via dephosphorylation, as in the case of the CHL1 transporter (Jung et al. 2009).

Fragmentary presentation of the molecular mechanisms ‘shaping’ root morphology demonstrates the complexity of plant response to nutrient stresses. To complete the picture of the function of particular genes expressed with up- or down-regulation, other very important elements of cell signaling, such as ethylene or ROS, should also be taken into consideration, both in response to stress caused by a deficiency in nitrogen, potassium or other nutrients (Shin and Schachtman, 2004; Jung et al. 2009; Hernández et al. 2012).

The search for genotypes effectively utilizing reduced levels of nitrogen or potassium fertilization in the early stages of plant growth and their incorporation into breeding programs (Low-Input) are currently one of the main areas in fundamental and applied research (Tuberosa and Salvi, 2007; Górny et al. 2011; Hoffmann et al. 2012). It has been demonstrated that plant response at the seedling stage is reflected in the reaction of an adult plant (Tuberosa et al. 2002).

Cultivated rye (*Secale cereale* var. *cereale* L.) is a source of genes determining tolerance to abiotic stresses. Rye is more productive than other cereals growing in acid, sandy and infertile soils. It has the highest tolerance to drought or nitrogen deficiency (Madej, 1996; Rzepka-Plevnes et al. 1997b; Rzepka-Plevnes and Kurek, 1997; Geiger and Miedaner, 2009).

Mapping populations, including populations of highly homozygotic RILs (recombinant inbred lines), enable the examination of such mechanisms when root architecture is subject to selection (Tuberosa and Salvi, 2007). Bulk segregant analysis conducted for pooled groups of segregants selected from such populations allows quick and effective identification of markers linked to QTL(s) controlled by major genes (Michelmore et al. 1991). The possibility of rapid screening of many loci (Michelmore et al. 1991; Darvasi and Soller, 1992, Darvasi and Soller, 1994; Angaji, 2009; Semagn et al. 2010), combined with the possibility of the application of markers exploring thus far unexamined chromosome regions, provides the possibility of the identification of a new range of variability as well as markers linked to the identified QTL(s) (Ye et al. 2005; Saleh, 2011; Smolik et al. 2012; Smolik, 2013b).

On the basis of excellent reviews characterizing the methods of bulk segregant analysis and selective genotyping, Darvasi and Soller (1992), Darvasi and Soller (1994), Gallais et al. (2007), Angaji (2009), and Semagn et al. (2010), emphasize overlapping methodological similarity, the role of description of allele frequency in pooled DNA samples, the kind of population, tail number and methodological limitations (Xu and Crouch, 2008). BSA has been successfully used in the mapping of single major genes (Barua et al. 1993; Villar et al. 1996; Quarrie et al. 1999; Shen et al. 2003), but a combination of BSA and the candidate genes approach could be very effective in discovering genes with a significant effect on QTLs (Angaji, 2009).

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Thus, the aim of this study was an application of the BSA method in the search in two phenotyped populations of RILs of rye for the molecular markers RAPD, ISSR and R-ISSR related with the extreme response of the plants on nutrition stress, and moreover a determination of their biological functions, conversion into SCAR markers and an attempt at the determination of their chromosomal localization.

MATERIALS AND METHODS

Plant materials

The research material consisted of two RILs populations of rye (F_9) derived from generation F_2 obtained from a combination of crossbreeding of inbred lines 153/79-1 x Ot1-3 (138 RILs) and Ot0-6 x Ot1-3 (191 RILs). These lines showed various responses to nutritional stress assessed at the seedling stage obtained in mature embryos cultures *in vitro*. The description of response of individual RILs to nutrient stress caused by simultaneous deficiency of nitrogen and potassium in a medium in *in vitro* mature embryo cultures for the population of [153/79-1 x Ot1-3]RILs (F_9) was presented in detail in a paper by Smolik (2013a). Phenotyping of response of the population of [Ot0-6 x Ot1-3]RILs (F_9) to nutrient stress was conducted with the use of the same methods and protocols as in a study by Smolik (2013a).

Morphological, physiological and biochemical response of seedlings of individual RILs in mature embryo cultures was assessed on modified MS mediums. The control medium -HNK, contained nitrogen and potassium at the dose of 6.00 mM and 2.00 mM, respectively, while the doses of other components were the same as in the MS medium. The LNK medium - assessing response of individual RILs to nutrient stress, apart from components typical of MS, contained 0.334 mM of N and 0.333 mM of K. Medium compositions were adopted according to Rzepka-Plevnes et al. (1997b). The experiment was carried out in stages, with simultaneous analysis of several RILs. Biometric, physiological and biochemical measurements were conducted after 11 days of the experiment. Morphological response of RILs was described with three parameters (CL: coleoptyle length; LRL: longest root length; RN: root number), physiological response - with seven parameters (total chlorophyll [Chl (*a* + *b*)], chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), Chl *a/b* and carotenoid (Car) contents were determined, and CO_2 assimilation rate (A) and transpiration rate (E) were measured), while biochemical response was presented with the use of three parameters by describing activity of oxidative stress enzymes: SOD (superoxide dismutase), CAT (catalase) and POX (peroxidase). The results were statistically analyzed with the use of multidimensional analysis of variance. The examined population was divided into groups of RILs with different response to nutrient stress with the Ward's agglomerative method (Euclidean distances) and also discriminant function analysis (DFA). On the basis of the obtained results, twenty RILs with extreme response to nutrient stress in each *in vitro* mature embryo culture were distinguished, and the lines were analyzed in the present study.

Each population under investigation was composed of: three inbred lines 153/79-1 (I_{21}), Ot0-6 (I_{23}), Ot1-3 (I_{23}) (Rzepka-Plevnes et al. 1997a), two hybrids F_1 (153/79-1 x Ot1-3 and Ot0-6 x Ot1-3), two pooled DNA samples (bulks) - tolerant and susceptible. They were composed of the DNA of twenty individual tolerant RILs and twenty susceptible to nutrient deprivation stress assessed at the seedling stage in mature embryos culture *in vitro*. These lines were also the subject of research according to the BSA method (bulk segregant analysis) presented by Michelmore et al. (1991). The selection of RILs for the bulks was performed on the basis of the results of statistical analyses conducted on a series of biometric, physiological and biochemical features describing the response of single RILs of rye to the stress caused by nitrogen and potassium deficiency, and for each population these are presented by Smolik (2013a). Two mapping populations were formed, each including 94 RILs, for the purposes of the genetic mapping of SCAR markers. DNA was extracted from fresh leaves using a DNeasy Plant Mini Kit (Qiagen, Germany) and was quantified using an Epoch unit and calculated using Gen5 software (BioTek, USA) to obtain 20-25 ng DNA pro 1 µl of DNA template.

RAPD and ISSR primers

The set of four hundred and twenty four RAPDs and one hundred and twenty ISSR primers were used in the research (Table 1).

Sequences of RAPD and ISSR primers were designed by the University of British Columbia - Canada (RAPD primer sets #1, 2, 3, 4 and 7; ISSR primer set #9) and synthesized by Sigma-Aldrich (Poland), each in 0.025 nmol scale.

PCR conditions

Amplification of RAPD and ISSR was conducted according to the protocols presented by Smolik (2012); Smolik (2013b) and Zietkiewicz et al. (1994), respectively. The papers by Smolik (2012) and Smolik (2013b) present combinations of primers RAPD+ISSR (R-ISSR) useful for the amplification of R-ISSR loci, and they were used in this study in the search for R-ISSR markers.

Electrophoresis and data analysis

PCR products were analyzed by electrophoresis (SubCell GT, Bio-Rad) in 1 x TBE buffer in 1.5% for RAPD and R-ISSR and in 2% agarose gels for ISSR (Basica LE GQT, Prona, Spain). GeneRuler 100 bp Plus DNA Ladder (3000-100 bp) (Thermo Scientific) was used as a size marker. The products were stained with ethidium bromide (0.5 µg ml^{-1}), visualized using MiniBIS Pro (DNR Bio-Imaging Systems, Israel) and G:Box/GeneSnap (Syngene, USA). Primers were selected in the first stage of the study, and RAPD as well as ISSR products were generated in reactions with them. For R-ISSR combinations, this stage was presented in two papers (Smolik, 2012; Smolik, 2013b). The research was conducted on pooled DNA samples (bulks), searching for products distinguishing 'tolerant' bulks from 'susceptible' ones in both the examined RILs populations (Michelmore et al. 1991, Masojć et al. 2009). Next, the examinations were repeated for selected primers adding DNA of the respective parental lines and F_1 hybrids. The following parameters were determined after this stage: number of primers generating amplicons, length of amplified products, number of amplified loci and generated amplicons (Table 1), and, moreover, number of mono-, polymorphic and genotype-specific products (Figure 1). The products differentiating pooled DNA samples were typified, and their origin was attributed to them (from one of thesecond inbred line). Presence (1) or absence (0) of each amplicons was scored for each genotype investigated. Matrices of similarity were generated using the PhylTool software (Buntjer, 2001) and were compared using Mantel (Daniel's) test statistics (Z) of significance (*XL*STAT). The relationship between the five rye genotypes were shown in the form of dendrograms produced by cluster analysis of the similarity coefficients using UPGMA. The bootstrap analysis was performed by TREECON using 2,000 pseudoreplications (Van de Peer and De Wachter, 1994). Once typed, based on an analysis of the fingerprinting of pooled DNA samples, amplicons potentially linked to the trait were analyzed on individual samples (RIL). Those products whose sequence considerably diverged from an expected distribution 1:1 (x^2- test) were accepted as significantly associated to the trait (Figure 2). These products were sequenced. PCR product extraction from the agarose gel was performed in order to sequence them using the Qiaquick Gel Extraction Kit (Qiagen, Hilden, Germany).

TOPO TA cloning

Products were cloned in the pCR2.1 vectors using TOPO TA cloning® kit (Invitrogen) and transformed into TOP10 competent *E. coli* cells according to the manufacturer's instructions. The transformants were selected according to the blue-white screening procedure. Plasmid DNA was purified from a 2 ml culture of individual colonies using the Plasmid Midi kit (A&A Biotechnology, Poland). Inserts sizes, after 5 min *Eco*RI restriction fast digest (Thermo Scientific), were checked using 1.0% agarose electrophoresis.

Sequencing and data analysis

Sequences were obtained using Beckman Coultier TDCS kit and run on a CEQ 8000 capillary sequencer according to the manufacturer's instructions. The sequences were edited and *assembled* into a full-length inserts sequence using BioEdit tool (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and were analyzed by searching for homology/orthology using the *blastn* and *blastx* algorithms by comparison with the NCBI GenBank database, and using data collected in the TIGR/TOGA/DFCI bases (http://www.jcvi.org/cms/research/projects/tdb/overview/) only for rye using BLAST (Altschul et al. 1990) and were presented in Table 2.

SCARs (Sequence Characterized Amplified Regions) were developed of by conversion of the selected RAPD, ISSR and R-ISSR markers. Pairs of specific primers and their nested variants were designed using Primer 3.0 software (Rozen and Skaletsky, 2000). The sequences of RAPD, ISSR and R-ISSR primers were identified in the resultant and adjusted sequences, and the sequences of specific pairs of primers were obtained by their elongation towards 3' of further 10-13 nucleotides. The synthesis of oligonucleotides was conducted in Genomed (Poland).

Genetic mapping

JoinMap 3.0 software was used for the construction of a genetic map framework of each of the examined RIL populations (Van Ooijen and Voorrips, 2001). The groups of conjugations for the identified SCAR and SCARs markers of known chromosomal localization were formed with critical values of LOD=3 describing their mutual segregations. The nomenclature of the presented markers was based on the nomenclature proposed by Schlegel and Korzun (2008), while for markers of the identified chromosomal localization for rye it was accepted according to their authors. Detailed information concerning origins and their authors is presented in Figure 4.

RESULTS

In the study, the usefulness of the search for molecular markers within two RIL populations of rye was described for 216 RAPD and 70 ISSR primers. In total, 2020 RAPD, and 696 ISSR amplicons were examined on average for each population, and 573 RAPD loci and 334 ISSR were described as polymorphic ones (Table 1, Figure 1).

The length of amplified products ranged from 2300 to 210 bp for RAPD, from 3090 to 210 bp for ISSR, and the number and character of the amplified loci depended on the examined genotype. Detailed results describing this stage of the research are presented in Table 1. Polymorphic products differentiating inbred lines were identified in RAPD, ISSR and R-ISSR profiles (Smolik, 2012; Smolik, 2013b). Their presence in an F_1 hybrid profile, as well as in various configurations in the profiles of two bulked DNA samples (bulks), both tolerant and susceptible to nutrient stress, are presented in Figure 1a.

Despite the fact that most PCR products differentiating inbred lines were noted in both bulk profiles, RAPD, ISSR and R-ISSR products specific both for inbred lines (*e.g.* potential donors of tolerance genes) and the respective bulk samples (bulks) were identified (Figure 1b). Analyzing dendrograms of similarity describing genetic relationships between inbred lines, F_1 hybrids and bulks of extremely different response to nutrient stress, it was demonstrated that the range of variability described using the RAPD and ISSR techniques is convergent (Figure 2). High bootstrap test values were noted for each of the clades presented, and high correlation coefficient values r(RAPD-ISSR) calculated using Mantel's test were observed for the matrices of genetic affinity (Figure 2).

Analyzing the dendrograms presented in Figures 2b, c, d, high genetic affinity was demonstrated between tolerant inbred lines (153/79-1 and Ot0-6) and tolerant bulks (data not shown). Similar relationships were observed for susceptible the inbred line Ot1-3 and susceptible bulks (Figure 2b, c, d). They were described differently for genotypes presented in Figure 2a.

The association of RAPD, ISSR and R-ISSR products, *i.e.* markers identified for inbred lines and differentiating bulked DNA samples with the examined trait, are presented as a distorted segregation (band:null) and differed significantly from the expected 1:1 ratio among twenty tolerant and twenty susceptible to nutrient stress RILs (Table 2).

Different segregation patterns were described. The first was the pattern describing the high frequency of allelic forms (markers) inherited from the line 153/79-1 or Ot0-6 and from the line Ot1-3, observed among tolerant and susceptible RILs, respectively. Such an effect for RILs of the 153/79-1 x Ot1-3 population was described for the following markers: *pr083_350; pr086_500; pr097_350* and *pr7_350* (Table 2, Figure 3). For RILs of the Ot0-6 x Ot1-3 population: *pr077_470*, *pr013_400*, *pr285_530* and *prs86+876_246* markers (Table 2).

The second pattern was characterized by distorted segregation, including a high frequency of allelic forms inherited from line 153/79-1 or Ot0-6 among tolerant RILs, and the frequency of these forms was consistent with the 1:1 ratio among susceptible RILs. This pattern of inheritance/segregation for RILs of the 153/79-1 x Ot1-3 population was described for the following markers: *pr177_530*, *pr083_600*, *pr086_900*, *pr857_460* and *prs86+876_246* (Table 2, Figure 3); for RILs of Ot0-6 x Ot1-3 population for *pr083_600*, *pr083_350*, *pr091_750*, *pr846_250*, *pr842_950*, *prs86+811 and prs85+836* markers (Table 2). The third type of distorted segregation was described for markers *pr077_470* and *pr824_1050* (RILs from population 153/79-1 x Ot1-3) and for one marker *pr013_400* (RILs from population Ot0-6 x Ot1-3) (Table 2). The origin of the examined markers, the sequences of specific primers designed during their conversion into SCARs and the length of specific products are presented in detail in Table 2.

Analyzing segregations of selected markers, attention was paid to their two different types with respect to the above. They concerned the markers *pr013_400* and *pr091_350,* for which a high frequency of allelic forms inherited from line 153/79-1 was observed among susceptible RILs selected from population 153/79-1 x Ot1-3, while their frequency among tolerant RILs did not differ from the expected 1:1 (Table 2).

Apart from three RAPD markers (*Xscszm*20, *Xscszm* 37 and *Xscszm* 50) and two ISSRs (*Xscszm*15 and *Xscszm*25), annotations of a very low *E-value* were identified for the remaining fifteen markers in GenBank, including unique sequences deposited in TIGR/TOGA/DFCI, homologous for genomes of rye, and also for barley and wheat (Table 3).

Among the markers contained in Table 3, of interest were those fitted for their sequence annotations for cytosolic acetyl-CoA carboxylase (Acc-2) (FJ436983.1 - *Xscszm17*; EU660895.1 - *Xscszm18*): HCBT-like putative defense response protein (AF446141.1 - *Xscszm21*), putative iron-deficiency specific for protein and putative ethylene-responsive TF (AP009567.1 - *Xscszm27*), HCBT-like putative defense response protein (AF446141.1 - Xscszm29), plastid acetyl-CoA carboxylase (Acc-1) genes (EU660897.1 - Xscszm49), as well as *Na* transporter HKT1;5 (*HKT1;5*) gene (DQ175869.1 - *Xscszm10 -* Aquaporin PIP1) (Table 3). Chromosomal localizations were either attributed to the selected markers, or they were presented as groups of linkage of currently unidentified affiliation (Figure 4).

DISCUSSION

With the exception of three RAPD markers and two ISSR, unique sequences that fitted to the genomes of wheat, barley and rye were identified for the remaining markers in GenBank. Homologies were attributed to a part of them, and significant alignments to sequences of selected chromosomes of unclear functions to the rest of them. Imprecise alignments identified in bases TIGR/TOGA/DFCI *stricte* for ESTs of rye (Table 3) may be explained by their generally low number collected for rye (ca. 10 thousands). For a comparison, for wheat and barley over 1.5 and 0.5 million ESTs sequences, respectively, are collected in GenBank or TIGR/TOGA/DFCI. Most of those presented in this study were derived from experiments on the construction of cDNA libraries of various genotypes of wheat and rye subjected to the activity of abiotic stress (Mochida et al. 2006). Alignments identified in much wider libraries of different genotypes (*T. aestivum* or *A. tauschii*) had similar functions.

According to Michelmore et al. (1991), Darvasi and Soller (1992), Darvasi and Soller (1994), Wang and Paterson (1994), Gallais et al. (2007) and Masojć et al. (2009), the significantly different frequency of DNA markers in the genetic profiles of extreme groups proves their connection with the examined trait, effectively identifying DNA regions related to it (Michelmore et al. 1991). The probability that the band/marker present in ten of one group (bulk), and absent in ten of a second group (bulk) is not linked to the trait amounts to 2×10^{-6} (Michelmore et al. 1991). Identification of such an arrangement in twenty genotypes forming two extreme bulks is highly improbable in terms of the quantitative features of the plants, such as their response to nutrition stress, preharvest sprouting or other edaphic stresses, which have been demonstrated in the results of the study by Masojć et al. (2009) and those presented in this study.

In a study on preharvest sprouting in rye, Masojć et al. (2009) divided markers characterized by distorted segregation in bulks into classes depending on the segregation described within the group and the parental line characteristics (gene donor). The authors proposed a genetic model describing rye response to preharvest sprouting attributing to them chromosomal localizations and QTLs regions.

An approach used in this study confirmed the usefulness of the description of frequencies of selected markers within more abundant bulks (Darvasi and Soller, 1992; Darvasi and Soller, 1994). Critical interpretation of distorted segregations *per se* as well as the sense of identified annotations was conducted for the purpose of examining extreme bulks formed from two identically phenotyped, *i.e.* having common parental components, highly homozygous and segregating RIL rye populations (Smolik, 2013a). An example may be markers *Xscszm36*, *Xscszm17* or *Xscszm25,* but also *Xscszm09, Xscszm37.* They differ in terms of their allocation to the classes, if the criteria presented by Masojć et al. (2009) for preharvest sprouting in rye are accepted. As may be concluded from this study, highly significant distorted segregation of the marker presented for one population may be a kind of over-interpretation in the description of the usefulness of particular markers for selection, unless their segregation in another population is determined. Such an approach bears the hallmarks of association analysis.

Michelmore et al. (1991) claim that when QTL is controlled by a few main genes, then the comparison of extreme segregants from the population enables quick identification of the main gene (genes), and thus the specific QTL.

Markers mentioned above, when analyzed separately, would be characterized by 'higher' linkage to examined traits. Analyzed collectively, they offer a critical contribution in results interpretation. The effect of such an approach is the selection of a few markers (*Xscszm09*, *Xscszm20* or *Xcszm36*), the localization of which in a region of specified QTL(s) is highly probable according to the opinion of Michelmore et al. (1991).

Sequences of identified markers are characterized by a significant matching to sequences of genes engaged in systemic plant response to nutrition stress and the pathway of C:N ratio regulation. Among them, both cytosolic acetyl-CoA carboxylase (Acc-2) (*Xscszm17* - FJ436983.1; *Xscszm18* - EU660895.1) and plastid acetyl-CoA carboxylase (Acc-1) (*Xscszm49* - EU660897.1) constitute an important element of plant response to various kinds of stresses, including nutrition. Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (Figueroa-Balderas et al. 2006). Malonyl-CoA is essential for fatty acid synthesis which occurs in plastids (Harwood, 1988). Fatty acids are required for elongated fatty acids and then for suberin or cuticle synthesis. Cytosolic malonyl-CoA is required for stilbene, and very long-chain fatty acid (VLCFA) synthesis (Ebel and Hahlbrock, 1977). VLCFA is required for the malonylation of 1-aminocyclopropane-1-carboxylic acid (ACC) - the precursor of ethylene (Liu et al. 1983; Figueroa-Balderas et al. 2006). ACC synthases produce ACC, which is converted into ethylene by ACC oxidases (Faris et al. 2001). According to Faris et al. (2001), Jung et al. (2009); Shin (2011), ethylene biosynthesis and ethylene signaling interact at the different levels with the plant's response to potassium, such as nitrogen deprivations, or up- or down-regulating the expression of their transcription factor genes. One such gene has been identified in this experiment as an annotation (AP009567.1 – 5e-127) for one molecular marker (SCAR) – *Xscszm27*.

An important role of cytosolic ACCase is the synthesis of flavonoids. High levels of ROS in the cell destructively affect DNA stability, and thus *i.a.* flavonoids may serve a key functional role in keeping the concentration of H_2O_2 at the sub-lethal level, in respect of their role as a signaling molecule involved in an increase in plant tolerance to *e.g.* nutrient stress, which at the phenotype stage is expressed in the elongation of roots (Yamasaki et al. 1997; Shin and Schachtman, 2004; Shin et al. 2005; Schachtman and Shin, 2007; Fini et al. 2011; Hernández et al. 2012).

Smolik et al. (2012) showed that fluorescence of dichlorofluorescein (DCF) in the elongating roots of seedlings of different RILs of rye, under nutrient starvation stress, was localized, as described for *Arabidopsis* by Shin et al. (2005), more in the epidermal than in the cortex zone. Hernández et al. (2012) presented identical observations of spatial localization of ROS in cytological preparations of tomato roots as Shin et al. (2005).

This fact, according to Shin et al. (2005), proves the mobilization of mechanisms playing a role in triggering gene expression during nutrient starvation, and may concurrently confirm the significant role of markers *Xscszm17, Xscszm18* or *Xscszm49* in molecular rye response to nutrition stress. Thus, the influence on diverse root morphology among tolerant genotypes such as those susceptible to N and K starvation stress, which was presented for RILs of rye by Smolik et al. (2012) and Smolik (2013a), may tentatively be attributed to functionally allelic variants of plastidsas well as cytosolic ACCases genes.

Marker *Xscszm17* was mapped on 5RL in both mapping populations. This localization was established based on linkage to chromosomally-specific markers *Xpm137*, *Xpm144* and *Xpsr-370* in population [153/79-1 x Ot1-3]RIL and to marker *Xpsr-370* in population [Ot0-6 x Ot1-3]RIL (Figure 4). Localization of these markers was identified and multiply verified on other genetic maps (Milczarski et al. 2007; Masojć et al. 2009, Myśków, 2010) and on a consensus map of rye (Milczarski et al. 2011).

In mapping population [Ot0-6 x Ot1-3]RIL marker *Xscszm18* was identified in the group showing linkage to markers *Xscszm21* and *Xscszm22* (Figure 4). Chromosomal localization was not attributed to them at this stage of the research. It may be only assumed that they are on the same chromosome as markers *Xscszm18*, *Xscszm21* and *Xscszm50*, and may constitute the region of specified QTL. Michelmore et al. (1991) showed that markers on both sides up to 25 cM from the targeted locus may be considered reliable. Those presented in Figure 4 as group B theoretically fulfill these criteria; however, they need practical verification.

Marker *Xscszm49* (plastid *Acc-1*), despite segregation consistent with the 1:1 distribution for mapping population [Ot0-6 x Ot1-3]RIL, did not form a group of linkage from other examined markers. It is interesting that in the hexaploid wheat genome the plastid gene (*Acc-1*) exists as a single copy, but the cytosolic (*Acc-2*) has more than one copy in each of the homologous chromosomes. These genes were mapped to the group 3 homologous chromosomes and to chromosome 5D, as was the case with rye, one of them on 5RL (*Xscszm17*).

The already mentioned putative iron-deficiency specific protein and putative ethylene-responsive transcription factor (ERFs) (*Xscszm27* - AP009567.1), similar to the previously mentioned SCARs, is also an important element of systemic plant response to stress. A single highly conserved AP2/ERF domain has been described for ERFs (Mishra et al. 2009). This domain binds to the GCC box present in the promoter region of stress responsive genes and may act as an activator or repressor of the expression of stress responsive genes in stress signal transduction pathways (Mishra et al. 2009). The potential of transcription factors lies in the fact that the same ERF can act in response to different stresses through the same or different signaling pathways. In addition, there are sets of genes, which respond to multiple stresses making it even more difficult to predict the exact role of ERFs (Jung et al. 2009). Such a transcription factor is RAP2.11 (Kim et al. 2012). Kim et al. (2012) in their study on *Arabidopsis* demonstrated that RAP2.11 binds to the GCC-box motif (GCCGGC) of promoter elements and directly up-regulates expression of *HAK5*.

Shin and Schachtman (2004), and later Jung et al. (2009), demonstrated that ethylene signaling plays a significant role in plant response to stress caused by K deficiency inducing ethylene production, and this in turn affects formation of ROS in the root hair differentiation zone, induces the expression of *HAK5* (high-affinity transporter), thus changing root morphology and plant tolerance to nutrient deprivation stress (Shin and Schachtman, 2004). However, this fact was not so certain a few years ago (Zaid et al. 2003).

It is interesting that this SCAR, despite the fact that a chromosomal localization was not attributed to it in either mapping population, formed a strict group of linkage with SCAR *Xscszm29* constituting an element of the sequence of the gene of the HCBT-like putative defense response protein, genes for P450 putative ankyrin (AP009567.1). In this study, this linkage was initially attributed to chromosome 1R together with locus TRX (Milczarski et al. 2007). It was not possible, however, to present it on a genetic map. The initial suggested linkage of locus TRX with loci *Xscsz429L950* and *Xscsz732L530* (Milczarski et al. 2007; Masojć et al. 2009) was rejected by a computer programme as incompatible with the accepted JoinMap3.0 algorithm. Taking into consideration the fact that SCARs *Xscsz429L950* and *Xscsz732L530*,which are specific for 1RL, formed a linkage group verified on other genetic maps of rye and did not form one with *Xscszm27 and Xscszm29*, it may only be supposed that these loci are located on 1RS.

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This suggestion is based on the results of the study presented by Bartoš et al. (2008), who described a homologue for HCBT-like putative defence response proteins also from *A. tauschii* among genes discovered in BAC end sequences (BES) from 1RS. This hypothesis also needs to be verified. Interesting may be the strong linkage between markers *Xscszm27* and *Xscszm29* identified in both mapping populations aligned to a putative iron deficiency sequence specific for protein and putative ethylene-responsive TF (5e-127) and HCBT-like putative defence response protein genes, respectively. Sequence *Xscszm27* is fully consistent with plant response to abiotic stress caused by deficiency of both N and K, but the sequence of marker *Xscszm29* is related to the whole spectrum of plant responses to pathogen attack (Brooks et al. 2002). In the combination presented in this study it may be interesting. Wide-ranging conclusions cannot be drawn with any certainty at this stage of the research. It should be however noticed that, in the case of iron deficiency in tomato, Graziano and Lamattina, (2007) noticed a significant increase in the accumulation of NO (nitric oxide). NO induces synthesis of phytoalexins catalyzed by HCBT-like proteins among which avenathramides (induced by NO presence) actively contribute to plant antioxidative response to stress, functioning as defence-signaling molecules, contributing to a lowering of ROS levels to below sub-lethal levels, as was the case with flavonoids.

An annotation identified in GenBank as a result of the matching to EST of a sequence of the marker *Xscszm10* as a sequence of barley transporter HvHKT1 encoded with gene *HKT1* is interesting in a context of segregation within extreme groups. However, in the base TIGR/TOGA/DFCI for rye it was matched to EST aquaropin PIP1 (DQ175869.1 - Sc01_05f04_A), and in the previous study as SCARECROW-like GRAS-family TF for *Leymus sp.*) (Smolik, 2012). Both HKT1 transporters and aquaporins (PIP1) have a significant relationship with plant response to stress, including nutrition stress (Maurel et al. 2008; Alemán et al. 2011).

During nutrient stress, K^+ is required for sucrose movements from shoot to root to provide root cell expansion in the soil (Schachtman and Schroeder, 1994; Alemán et al. 2011). HKT1 is engaged in high-affinity uptake of Na^+/K^+, and its expression is observed in the roots (Schachtman and Schroeder, 1994). The transport mechanism for both Na^+ and K^+ led by wheat transporter HKT1 has not yet been identified (Schachtman and Schroeder, 1994; Alemán et al. 2011). Aquaporins play a central role in plant water relations (Johansson et al. 2000). PIP1 and -2 are plasma membrane intrinsic proteins. They have also been linked to plant mineral nutrition and carbon and nitrogen fixation (Maurel et al. 2008). It may be concluded from the study of Barthes et al. (1996) that NO_3^- is engaged in the transcription control of aquaporins, and down regulation of PIPs is observed in the case of its deficiency, as in the case of K^+ deficiency (Maurel et al. 2008). During drought, strong PIP induction was observed, leading to optimized residual soil water uptake. Moreover, aquaporins, *i.e.* PIP, are able to transport CO_2 serving as pores in leaves, and importantly they transport N compounds (*i.e.* urea) during N remobilization (the first part of the response to nutrient stress) from the vacuole (Maurel et al. 2008).

Referring to the role of root plasma membrane in plant systemic response to nutrient stress caused by deficiency of *e.g.* potassium, one should present the results of research by Nieves-Cordones et al. (2008). The authors demonstrated that K^+ starvation treatment causes more-negative - ca -210 mV (hyper-polarized), potential of membrane in plant roots and results in increased expression of *LeHAK5* when the concentration of K^+ in roots decreases.

The molecular response of rye RILs to nutrient stress presented in the form of biological interpretation of various ESTs aligned to identified RAPD, ISSR and R-ISSR markers, as a result of the application of the bulk segregant analysis method (Michelmore et al. 1991), is compliant with the results of the study presented *i.a.* by Huang et al. (2004), Lian et al. (2005), An et al. (2006), Liu et al. (2008), and Hoffmann et al. (2012) for the root architecture features of various plant species examined in conditions of nutritional stress with the application of a classical analysis of quantitative features (QTLs).

Assessment of response of root architecture to stress caused among other things by nitrogen, potassium or phosphorus deficiencies is conducted in early growth stages (seedling stage) under laboratory conditions (aeroponics, pots, filter-paper rollers, gel chambers).

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They are treated as alternative and complementary to research on mature plants in field (Messmer et al. 2011; Tuberosa et al. 2011). Tuberosa et al. (2011) emphasize that each laboratory method has a range of limitations and needs to be verified in field experiments.

The potential of *in vitro* screening and selection of plants were excellently reviewed by Bright (1991). Description of relationships between the phenotypic response of seedlings to nutrient deprivation of N and K with the use of a mature embryo culture in a gel chamber, agar plates, glass tubes has some negative sides. They include the possibility of reduction of mineral availability, which may result from physiochemical characteristics of used agar (Beruto et al. 1999a, Beruto et al. 1999b). Jain et al. (2009) state that, for instance, different agar brands may have varied nutrient diffusion rate, elemental and organic impurities and gel strength. The authors suggest that the characteristics may modify the phenotypic response of examined genotype depending on the composition of used medium, which makes some components of the medium unavailable to plants. Other factors affecting seedling growth in a mature embryo culture may include embryo length (Monnier, 1990) and C:N ratio (Malamy and Ryan, 2001; Mills, 2009).

However, there are many reports in literature demonstrating that evaluation of root architecture in response to *e.g.* nutrient stress (-N, -K), carried out under laboratory conditions (hydroponics, *in vitro* culture, gel chambers, agar plates), is reflected in the plant mature stage (Tuberosa and Salvi, 2007; Tuberosa et al. 2011; Messmer et al. 2011; Liu et al. 2013).

Most precisely, the aim may be summarized by the statement presented in the study of Liu et al. (2008) who conclude that QTLs for root architecture (*i.a.* root length, root number) of the seedlings of tolerant genotypes examined inter alia in laboratory conditions with low levels of N or K are consistent with QTL localization for grain yield, N uptake, NUE (nutrient use efficiency) confirming that these traits constitute significant criteria of genotype selection for low-input systems (Tuberosa et al. 2002; An et al. 2006; Liu et al. 2008; Górny et al. 2011; Hoffmann et al. 2012). Detailed references to QTLs described for root architecture at seedling stage and traits of mature plant in field conditions may be found in the studies of Liu et al. (2008) and Hoffmann et al. (2012). A range of QTL associations to roots traits and plant response to drought and to phosphorus deficiency have been described in maize (Hund et al. 2004; Mano et al. 2005; Zhu et al. 2006). Agrama and Moussa (1996), Gallais and Hirel (2004), Ribaut et al. (2007) and Liu et al. (2008) identified a range of QTLs for the features linked to N assimilation and NUE, including 5 and 6 QTLs for grain yield in the conditions of low (LN) and high (HN) levels of N fertilization. Ribaut et al. (2007) described a few identical QTLs associated probably with maize tolerance to drought and low N fertilization.

In summary it should be concluded that plant requirements for N or K are fulfilled by their effective uptake, and in the case of deficiencies by re-arrangement of plant genomes that suitably adjust their metabolism to changing conditions (Shin and Schachtman, 2004; Krouk et al. 2010; Tsay et al. 2011; Smolik, 2013a). Active response to nutrition stress is a result of the root architecture and efficiency of mechanisms re-mobilizing or allocating assimilates to the roots. Sequences of RAPD markers, ISSR and probably for the first time R-ISSR identified in this study and when compared to sequence ESTs confirmed that the differences between tolerant and susceptible RILs may be expressed in the form of allelic functional variants of genes encoding *i.a.* acetyl-CoA oxidase or putative ethylene-responsive transcription factors (ERF). The differences may be significant since they are often subjected to regulation by miRNA (Kuo and Chiou, 2011; Shin, 2011; Schachtman, 2012), and for genes engaged in N uptake and assimilation by nitrate responsive *cis*-acting elements (Hu et al. 2009).

Further study is required in order to complete the construction of genetic maps, localization of non-mapped markers and identification of new markers for MAS. This might occur within an examination of the expression of genes engaged in the sensing and signaling of N/K deficiency.

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Tables

Table 1. Characteristics of generated RAPD and ISSR products for parental inbred lines, hybrids and bulks of two investigated populations of RILs of rye.

Population/Genotype		RAPD						ISSR					
		Primers and loci amplified number	Amplicons size range (bp)	Total/mean loci amplified				Primers and loci amplified number	Amplicons size range (bp)	Total/mean loci amplified			
				Total loci amplified	Monomorphic	Polymorphic	Genotype-specific			Total loci amplified	Monomorphic	Polymorphic	Genotype-specific
153/79-1 x Ot1-3	153/79-1	216 - 1989	2240-210	1592	1305 (66%) - 6	640 (32%) - 3	44 (2%) - 0.2	69 - 696	2960 - 220	477	305 (44%) - 4.4	377 (54%) - 5.5	14 (2%) - 0.2
	Bulk - tolerant			1663						567			
	F1			1668						611			
	Bulk - susceptible			1660						561			
	Ot1-3			1603						534			
Ot0-6 x Ot1-3	Ot0-6	215 - 2052	2300-230	1644	1479 (72%) - 6.9	506 (25%) - 2.3	67 (3%) - 0.3	70 - 697	3090 - 210	559	394 (57%) - 5.6	291 (42%) - 4.3	9 (1%) - 0.01
	Bulk - tolerant			1772						633			
	F1			1798						610			
	Bulk - susceptible			1772						594			
	Ot1-3			1587						540			

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Table 2. Sequences of SCARs designed on the basis of the conversion of selected RAPD, ISSR and R-ISSRs, together with their segregation/distorted in bulks of RILs of rye of two rye populations considered as tolerant and susceptible to nutrient stress assessed in the seedling stage in *in vitro* mature embryos cultures.

Screening primer and product length (bp)	Source	153/79-1 × Ot1-3						Source	Ot0-6 × Ot1-3						SCAR	Sequence of SCAR primers 5’ 3’	SCAR - product length (bp)
		Tolerant		x^2 (1:1)	Susceptible		x^2 (1:1)		Tolerant		x^2 (1:1)	Susceptible		x^2 (1:1)			
		band	null		band	null			band	null		band	null				
RAPD																	
177_530	153/79-1	15	5	5.00*	8	12	0.80	-							*Xscszm16*	F: GAGCACCAGGACACGACGTA R: AGCACCAGGGTCAGATGG	530
083_350	153/79-1	15	5	5.00*	4	16	7.20**	Ot0-6	17	3	9.80**	10	10	0.00	*Xscszm17*	F: GGGCTCGTGGCACTAGTAA R: GGGCTCGTGGGACTCATCA	350
091_350	153/79-1	15	5	5.00*	8	12	0.80	Ot0-6	9	11	0.20	11	9	0.20	*Xscszm18*	F:GGGTGGTTGCCCAATATCCC R: GGGTGGTTGCCAAGCAGCAG	370
091_750	153/79-1	8	12	0.80	15	5	5.00*	Ot0-6	15	5	5.00*	8	12	0.80	*Xscszm19*	F: GGGTGGTTGCCAAACCGGG R: GGGTGGTTGCGCAGCCATT	750
077_470	Ot1-3	6	14	3.20	15	5	5.00*	Ot1-3	6	14	3.20	17	3	9.80**	*Xscszm20*	F: GAGCACCAGGTCAATCTAGAA R: GAGCACCAGGCGGTGAGA	470
083_600	153/79-1	16	4	7.20**	9	11	0.20	Ot0-6	17	3	9.80**	10	10	0.00	*Xscszm21*	F: GGGCTCGTGGCTACATAGTCG R: GGGCTCGTGGGGAACGCG	600
285_530	-							Ot0-6	15	5	5.00*	5	15	5.00*	*Xscszm22*	F: GGGCGCCTAGGCACAGCC R: GGGCGCCTAGTCGTAGCAAC	530
086_500	Ot1-3	7	13	5.00*	15	5	5.00*	Ot1-3	9	11	0.00	16	4	7.20**	*Xscszm36*	F: GGGGGGAAGGTGGGAAA R: GGGGGGAAGGTATGTACGTATAGTT	500
013_400	153/79-1	4	16	7.20**	16	4	7.20**	Ot0-6	8	12	0.80	3	17	9.80**	*Xscszm37*	F: CCTGGGTGGATACCACAGCA R: CCTGGGTGGACTGATCATGATT	400
086_900	153/79-1	16	4	7.20**	11	9	0.20	-							*Xscszm50*	F: GGGGGGAAGGAGGTGGG R: GGGGGGAAGGTTGTACGTATAGTT	490
ISSR																	
842_950	-							Ot0-6	15	5	5.00*	10	10	0.00	*Xscszm15*	F: GAGAGAGAGAGACCCGGGCCC R: GAGAGAGAGAGACCCGGGCGC	950

st.7_350	153/79-1	15	5	5.00*	3	17	9.80**	Ot0-6	11	9	0.20	10	10	0.00	*Xscszm25*	F: CACACACACACACAGGCAAGCA R: CACACACACACACAGGGAGGG	350
824_1050	Ot1-3	8	12	0.80	15	5	5.00*	Ot1-3	9	11	0.20	12	8	0.80	*Xscszm27*	F: TCTCTCTCTCTCTCTCGTAGCC R: TCTCTCTCTCTCTCTCGTGTTCTT	1050
824_1150	153/79-1	12	8	0.80	11	9	0.20	Ot0-6	16	4	7.20**	8	12	0.80	*Xscszm29*	F: TCTCTCTCTCTCTCTCGTAGCCT R: TCTCTCTCTCTCTCTCGTGTTCTT	1030
846_250	153/79-1	4	16	7.20**	12	8	0.80	-							*Xscszm48*	F: CACACACACACACACAGTTTGTA R: CACACACACACACACAATTTTCC	250
846_250	-							Ot0-6	15	5	5.00*	9	11	0.20	*Xscszm49*	F: CACACACACACACACAGTTTGTAAG R: CACACACACACACACAAATTTTCC	250
R-ISSR																	
86+811_468	-							Ot0-6	15	5	5.00*	11	9	0.20	*Xscszm01*	F: GAGAGAGAGAGAGAGACAGCCA R: GGGGGGAAGGGGAGGCG	468
86+876_246	153/79-1	15	5	5.00*	9	11	0.20	Ot0-6	15	5	5.00*	5	15	5.00*	*Xscszm09*	F: GATAGATAGACAGACACCCGC R: GGGGGGAAGGGAGGGGA	246
85+836_720	-							Ot0-6	15	5	5.00*	7	13	9.80**	*Xscszm10*	F: AGAGAGAGAGAGAGAGTAGGAGA R: GTGCTCGTGCCATACGCCA	720

* Significant at $P < 0.05$, ** at $P < 0.01$.

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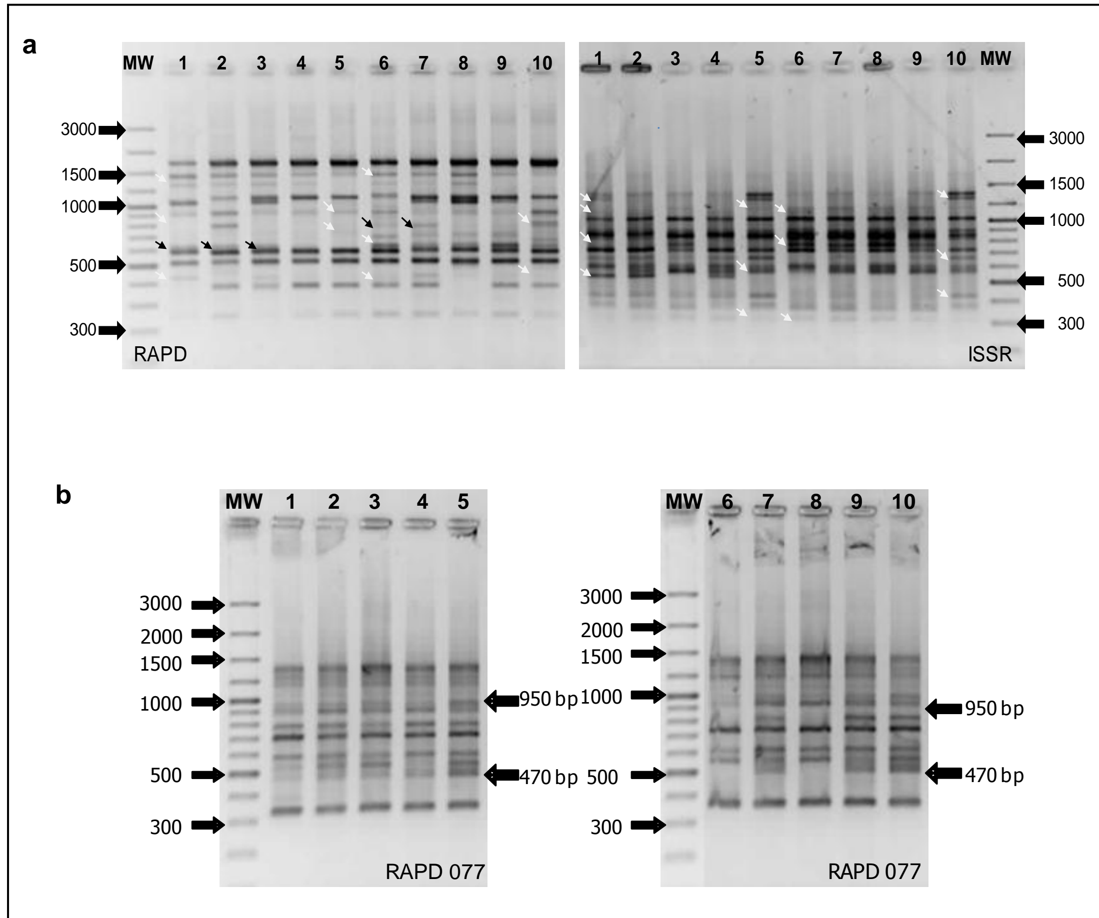
Table 3. List of annotations identified in the NCBI GenBank and TIGR/TOGA/DFCI databases as a result of alignment of obtained sequences to nucleotide and transcript sequences available in the databases, including ESTs identified for rye.

SCAR	GenBank *E*-value Accession	Annotation	TIGR/TOGA/DFCI[1] *E*-value Accession	Annotation
Xscszm16	FN564428.1 4e-16 *T. aestivum*	3B-specific BAC library	Sc02_12b04_A (0.94)	putative uncharacter.protein
Xscszm17	FJ436983.1 7e-46 *T. aestivum*	BAC 1354M21 cytosolic (Acc-2)	BE496197 6e-36 *T. aestivum*	chrom. undeterm. SCAF14724
Xscszm18	EU660895.1 4e-19 *T. aestivum*	cytosolic (Acc-2), put. amino acid permeases genes		
Xscszm19	AC118133.4 6e-80 *O. sativa*	chromosome 3 BAC OSJNBb0016H12 genomic sequence		
*Xscszm20**	no significant annotation		Sc02_11g09_A (0.60)	putative uncharacter.protein
Xscszm21	AF446141.1 3e-18 *A. tauschii*	HCBT-like put. defense response protein		
Xscszm22	FN645450.1 3e-116 *T. aestivum*	chrom. 3B-specific BAC library, contig ctg0011b	CD897568 2e-23 *T. aestivum*	no annotation
Xscszm36	AK367698.1 1e-99 *H. vulgare*	Cf2/Cf5 disease resist. protein homolog (Big1)	Sc02_12h11_A (0.51)	putative uncharacter.protein
*Xscszm50**	no significant annotation		Sc02_09c12_A (0.91)	putative uncharacter.protein
Xscszm27	EU338878.1 5e-97 *S. cereale*	clone 498_I-F1	Sc01_03h11_A (0.54)	no annotation
	AP009567.1 5e-127 *H. vulgare*	putative iron-deficiency specific for protein and putative ethylene-responsive TF	CA019942 7e-43 *H. vulgare*	no annotation
Xscszm29	AF446141.1 0.00 *A. tauschii*	HCBT-like putative defense response prot.	AJ611326 2e-82 *T. turgidum*	no annotation
	AB298185.1 0.00 *T. aestivum*	genes for P450, putative ankyrin		
	AP009567.1 7e-169 *H. vulgare*	putative iron-deficiency specific for protein and putative ethylene-responsive TF		
Xscszm48	EU660897.1 1e-37 *T. aestivum*	clone BAC 122F14 plastid (Acc-1) gene	CJ633403 9e-19 *T. aestivum*	Mochida et al. (2006)
Xscszm49	EU660902.1 1e-37 *A. tauschii*	BAC RI43D6 plastid acetyl-CoA carboxylase (Acc-1) genes	CA699415 5e-06 *T. aestivum*	Mochida et al. (2006)
Xscszm01	AK251555.1 3e-44 *H. vulgare*	mRNA for predicted protein	TA76746_4565 4e-19 *T. aestivum.*	hypothetical protein P0486C01.15
Xscszm09	BU971038.1 4e-19 *H. vulgare*	clone HB16G22 5-PRIME	Sc01_03h01_A (0.90)	
Xscszm10	DQ175869.1 4e-56 *H. vulgare*	*Na* transporter HKT1;5 (*HKT1;5*) gene	Sc01_05f04_A (9e-13)	aquaporin PIP1

*Markers *Xscszm20* and *Xscszm50* are listed in Table 3 due to identified matching to the sequences deposited in ESTs base for rye, despite the fact that no significant matching was identified in GenBank.

[1]TIGR: The Institute for Genomic Research; TOGA: TIGR Orthologous Gene Alignments; DFCI: Dana Farber Cancer Institute.

Figures



MW: Molecular Weight; 1: 153/79-1; 2: bulk tolerant; 3: F_1; 4: bulk susceptible; 5: Ot1-3; 6: Ot0-6; 7: bulk tolerant; 8: F_1; 9: bulk susceptible; 10: Ot1-3.

Fig. 1 (a) Electrophoregrams of selected RAPD and ISSR products generated in the experiment in order to search for differences between DNA bulks in comparison with genetic profiles of inbred lines and hybrids. White arrows denote selected amplicons present in the profiles of parental lines and in different combinations in F_1 hybrid(s) and bulks. Black arrows denote amplicons differentiating DNA bulks. (b) Electrophoregrams and selected RAPD products present in parental line Ot1-3, absent in 153/79-1 and Ot0-6. Segregations of these products are presented in Figure 4.

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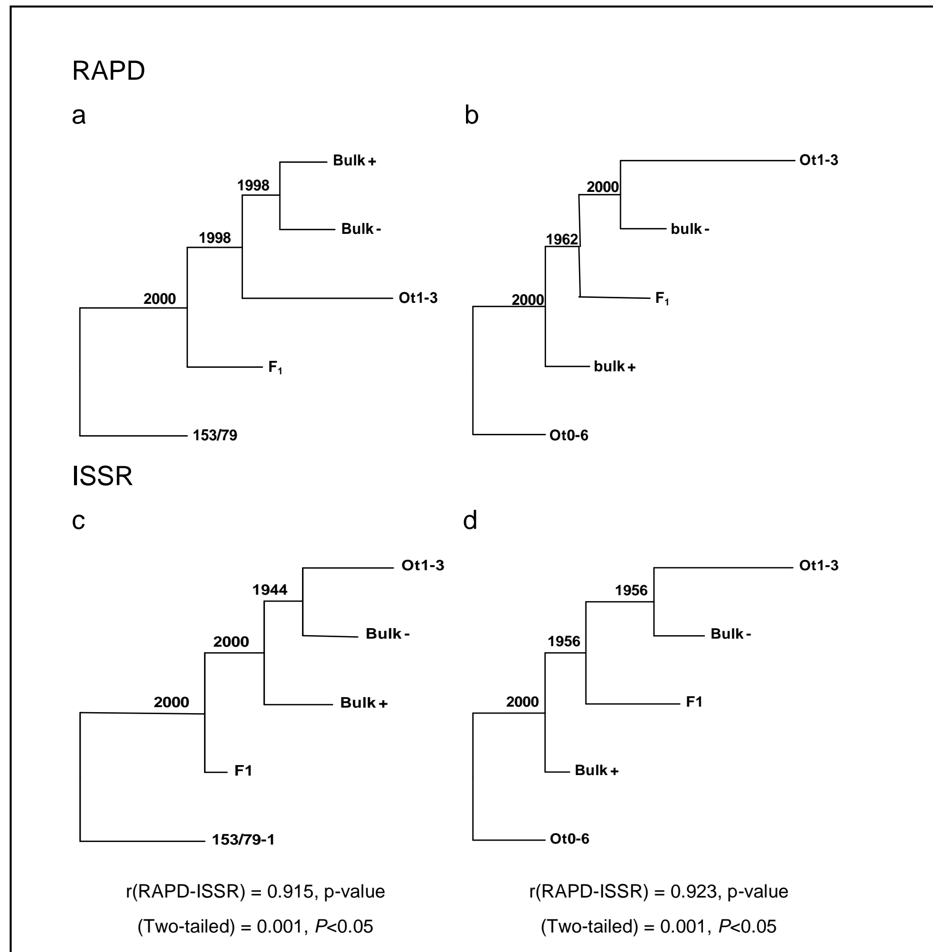

Fig. 2 Dendrograms of genetic similarity drawn for inbred lines, F_1 hybrids and DNA bulks of two rye populations ('a' and 'c' - [153/79-1 x Ot1-3]RIL; 'b' and 'd' - [Ot0-6 x Ot1-3]RIL), generated from DNA of single inbred lines respectively tolerant (+) and susceptible (-) to nutrient stress at the seedling stage, examined in the study. Correlation coefficient r(RAPD-ISSR) between matrices of genetic similarity, calculated with the Mantel test and obtained for the examined genotypes with the use of respectively RAPD and ISSR method, is presented below the dendrograms. The numbers above branches indicate value of bootstrap in which the branch was observed in 2,000 pseudoreplications.

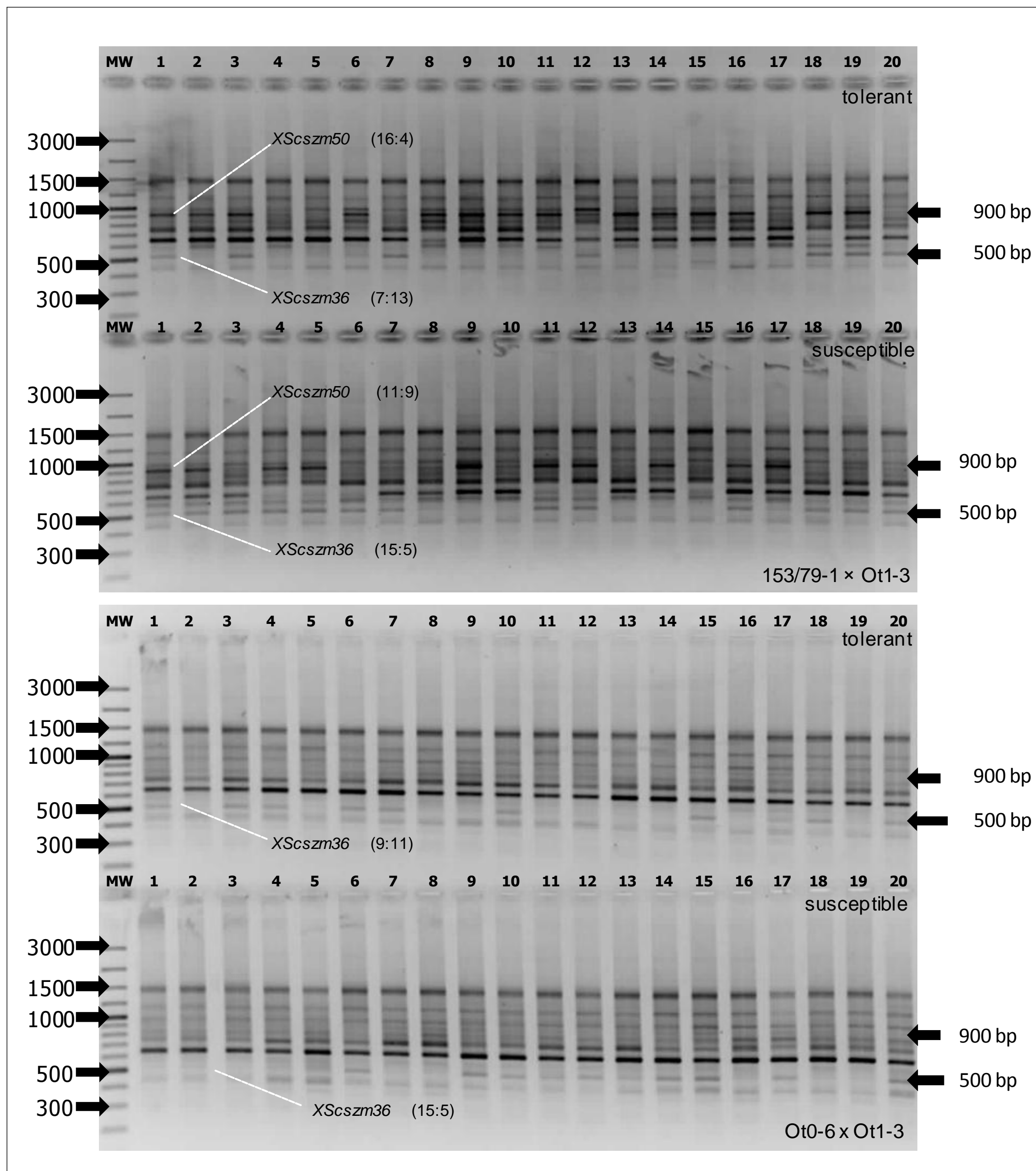

Fig. 3 Electrophoregrams of RAPD products generated for single RILs from bulks with extremely different response to nutrient stress determined at the seedling stage in *in vitro* cultures of mature embryos. *Xscszm* denote RAPD products converted into SCAR-type markers. Prevalence of RAPD products (900 and 500 bp long) determined in groups of RILs and significance of deviations from 1:1 segregation (band:null) for products denoted with *Xscszm* were calculated using the x^2 test. The value of the test and *p*-values are given in Table 2.

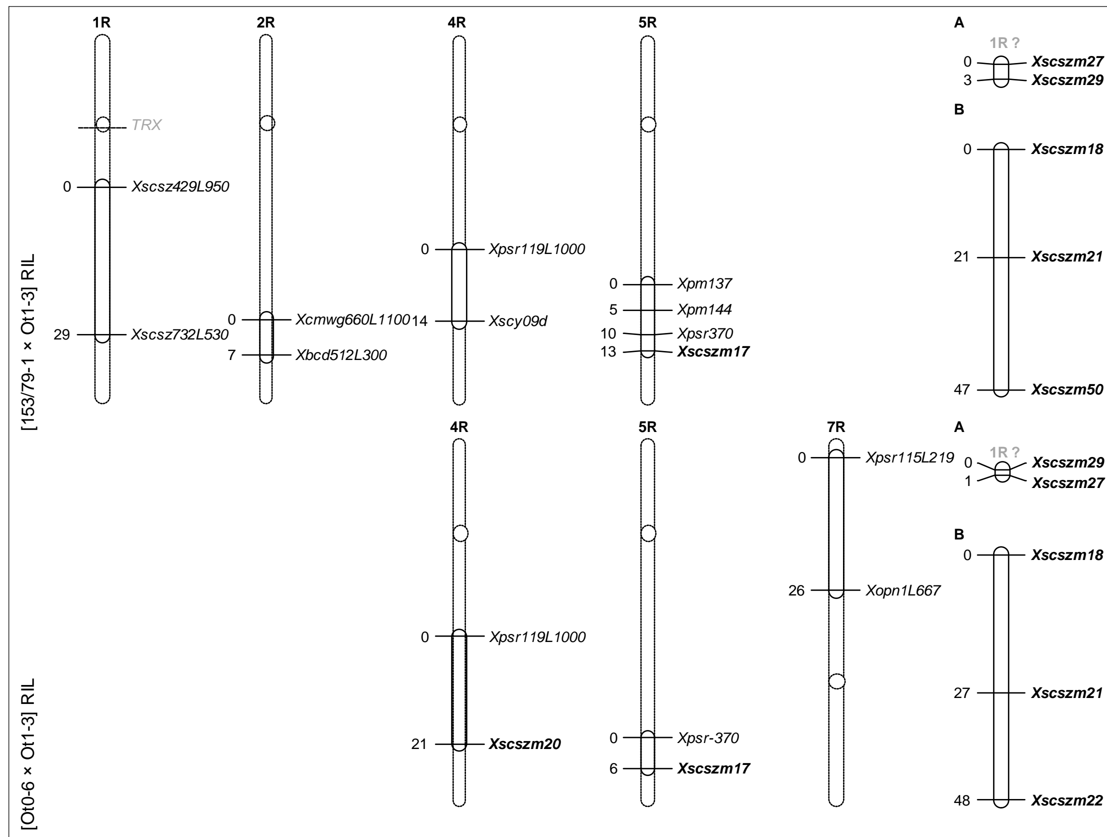

Fig. 4 Linkage groups of RAPD, ISSR and R-ISSR markers and chromosomal localization of selected SCARs in populations 153/79-1 x Ot1-3 and Ot0-6 x Ot1-3. Groups of linkages for markers RAPD, ISSR and R-ISSR identified and converted to SCARs (in bold) during the course of the completion of the study were presented on the background of R chromosomes, which together with approximate centromere localizations are marked with dotted lines. These localizations include markers STS markers - TrxL1100 (1R - K - Milczarski et al. 2007); *Xpsr119L1000* (4R - K, L, M, S Milczarski et al. 2007); *Xpsr106.1L350* (6R - K Milczarski et al. 2007); *Xbcd512L250* and *Xcmwg660L1100* (2R - K, L - Milczarski et al. 2011); *Xscy09d* (4R - K, L, S - Stracke et al. 2003); SCAR markers - *Xscsz429L950* (1R - K, L, S Stojałowski has designed specific primer pairs); *Xscsz732L530* (1R - K, S Milczarski et al. 2007); *Xopn1L667* (7R - S Matos et al. 2005); *Xopq4L578* (7R - K, S NCBI-AY587511 - Milczarski P. has designed specific primers pair), *Xpm137, Xpm144* and *Xpsr-370* (5R - Masojć et al. 2009; conversion of RAPD markers into SCAR has been made by Owsianicki R.) verified by various authors and on various rye mapping populations (marked with capitals by chromosome number). A and B - groups of linkages of an untraced chromosomal localization.