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ORIGINAL PAPER

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Targeting the aluminum tolerance gene *Alt3* region in rye, using rice/rye micro-colinearity

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Abstract Characterization and manipulation of aluminum (Al) tolerance genes offers a solution to Al toxicity problems in crop cultivation on acid soil, which composes approximately 40% of all arable land. By exploiting the rice (*Oryza sativa* L.)/rye (*Secale cereale* L.) syntenic relationship, the potential for map-based cloning of genes controlling Al tolerance in rye (the most Al-tolerant cereal) was explored. An attempt to clone an Al tolerance gene (*Alt3*) from rye was initiated by using DNA markers flanking the rye *Alt3* gene, from many cereals. Two rice-derived, PCR-based markers flanking the *Alt3* gene, B1 and B4, were used to screen 1,123 plants of a rye F_2 population segregating for *Alt3*. Fifteen recombinant plants were identified. Four additional RFLP markers developed from rice genes/putative genes, spanning 10 kb of a 160-kb rice BAC, were mapped to the *Alt3* region. Two rice markers flanked the *Alt3* locus at a distance of 0.05 cM, while two others co-segregated with it. The rice/rye micro-colinearity worked very well to delineate and map the *Alt3* gene region in rye. A rye fragment suspected to be part of the *Alt3* candidate gene was identified, but at this level, the rye/rice microsynteny relationship broke down. Because of sequence differences between rice and rye and the complexity of the rye sequence, we have been unable to clone a full-length candidate gene in rye. Further attempts to clone a full-length rye *Alt3* candidate gene will necessitate the creation of a rye large-insert library.

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Introduction

Aluminum (Al) toxicity is one of the major constraints on world crop cultivation in acid soils. The use of Al-tolerant cultivars could provide an efficient, stable genetic solution to this problem. An understanding of Al tolerance mechanisms in cereals is important for the development of Al-tolerant cultivars; however, Al tolerance mechanisms, especially at the molecular level, are not well understood, owing to a lack of analytical tools to dissect the phenomena underlying the Al tolerance variation among cereal species. Knowledge of Al-responsive or Al-regulated genes has accumulated from many different perspectives and species (Matsumoto 2000; Ezaki et al. 2001; Rodriguez Milla et al. 2002; Sasaki et al. 2004); however, as yet, the gene or genes directly responsible for Al tolerance in plants have not been identified and characterized. Sasaki et al. (2004) have characterized a malate transporter from wheat (*Triticum aestivum* L.) that is constitutively expressed in wheat root apices and was shown to increase Al tolerance of tobacco (*Nicotiana tabacum*) cells. Cloning a gene responsible for Al tolerance and analyzing the genetic mechanism of its action in cereals would considerably advance our ability to manipulate Al tolerance.

The development of DNA markers and a corresponding linkage map has facilitated map-based gene isolation, based on a recognizable phenotype and the genomic location of its determinant without knowing the characteristics of the desired gene (Collins 1992). Unfortunately, the application of map-based cloning in plants with large and complex genomes, such as rye, has been difficult because of the impracticality of chromosome walking and the paucity of tools to develop

high-density genetic and physical maps in such species. In rye, map-based cloning is considerably more difficult because of the lack of a large-insert DNA library.

The long arm of chromosome 4 (4RL) in rye contains an Al tolerance gene, *Alt3* (Miftahudin et al. 2002, 2004), which could be a novel gene responsible for controlling Al tolerance in cereals. Comparative mapping between rice (*Oryza sativa* L.) and members of Triticeae, such as wheat and barley (*Hordeum vulgare* L.), and the availability of the rice genome sequences could be of considerable benefit in dissecting out the *Alt3* gene in rye. By exploiting the strong syntenic relationship between rice and rye (Miftahudin et al. 2004), a high-resolution map of the *Alt3* gene region in rye could be constructed, and the *Alt3* gene region could be delimited. Once a candidate orthologue of *Alt3* has been identified from that region, it could be used to tag the gene in rye, which would eliminate the difficult and time-consuming task of developing a rye large-insert library. This paper reports our effort to target the Al tolerance gene (*Alt3*) region in rye, using micro-colinearity between rice and rye.

Materials and methods

Mapping populations

A rye F_2 population consisting of 1,123 plants developed from the cross between Al-tolerant (M39A-1-6) and Al-sensitive (M77A-1) parents was used. Those were the same parents used to develop a rye F_6 recombinant inbred line (RIL) population (Miftahudin et al. 2002). In addition, F_3 families derived from recombinant F_2 plants involving the *Alt3* region were also utilized.

DNA isolation and screening for recombinant plants

For PCR analysis of the F_2 population, DNA was isolated from 10 cm to 15 cm of fresh leaf tissue from individual 2-week-old plants, using a rapid DNA isolation technique previously described (Miftahudin et al. 2004). For Southern hybridization of probes to selected recombinant F_2 plants, DNA of an appropriate quality was isolated from individual plants, following the standard CTAB method (Saghai-Maroof et al. 1984). The two PCR-based markers, B1 and B4, which were known to flank the *Alt3* gene (Miftahudin et al. 2004), were used to identify plants having recombination events involving both markers in the *Alt3* region. DNA from each of the 1,123-member F_2 population was used as a template for PCR using the primer pairs of each marker.

RNA isolation and Northern hybridization

For the time-course experiments involving gene expression, several seedlings were used for RNA isolation. To eliminate any plant-to-plant variation, seedlings were grown, and only roots of similar length were selected. Total RNA from root tips of unstressed and stressed plants was isolated using TRIzol reagent (Invitrogen, Carlsbad, Calf.), following manufacturer's protocol. Equal amounts of total RNA (10 μg) were electrophoresed in a 1.5% agarose–formaldehyde gel, transferred to nylon membranes (Hybond-N^+, Amersham Pharmacia, Piscataway, N.J.), and then fixed by UV cross-linking (Fisher, Pittsburgh, Pa.). Blots were immersed briefly in a methylene blue staining solution (0.3 M sodium acetate, pH 5.2; 0.6% methylene blue), and rinsed in deionized distilled water. The moist blots were wrapped in clear plastic and scanned. Probes were obtained by PCR amplification or restriction digestion with appropriate enzymes. The DNA was purified using a Geneclean kit (Bio 101, Carlsbad, Calif.), and 50–100 ng were labeled with α-[^{32}P] dCTP, using DNA Polymerase I Klenow (Promega, Madison, Wis.). Probes were denatured at 95°C before adding to the hybridization solution. Pre-hybridization (8 h) and hybridization (36 h) were carried out at 65°C in 7% SDS, 0.191 M Na_2HPO_4, 0.058 M NaH_2PO_4, 1% BSA, and 100 μg/ml denatured salmon sperm DNA. Washes were done at room temperature with 2× SSC, 0.5% SDS and, for those blots showing stronger signals, with 0.1× SSC, 0.1% SDS at 65°C. Membranes were exposed to X-ray films at −80°C.

Phenotypic analysis of $F_{2:3}$ lines

From each F_2 plant identified as being recombinant in the *Alt3* region by the primer pairs B1 and B4, 20–38 plants per F_3 family were screened for Al tolerance at 2 ppm of Al, using the techniques previously reported (Somers et al. 1996; Miftahudin et al. 2002). Seeds were germinated at 4°C for 24 h, followed by incubation at room temperature for 12 h until the seedlings had an average root length of about 5 mm. The seedlings were then placed on minimal nutrient solution (Miftahudin et al. 2002) in a growth chamber (25°C) for 24 h. The nutrient solution was replaced with fresh minimal nutrient solution containing 2 ppm of Al for another 24 h. After rinsing the roots in ultra-pure water, the roots were placed in 1% Eriochrome-R solution for 10 min at room temperature. The roots were then rinsed three times in ultra-pure water before the seedlings were returned to fresh minimal nutrient solution without aluminum and grown for 48 h in a growth chamber. The length of root from the red Eriochrome-R stain mark to the root tip was measured on each of the three longest roots from every plant and the average length calculated. Plants that exhibited root re-growth of 2.5 cm or less were classified as sensitive; all others were classified as tolerant plants. The segregation pattern of each family was evaluated by using a chi-square test to determine the Al tolerance genotype of the F_2 recombinant plants.

Development of RFLP markers from a rice BAC clone

A genetic distance of 0.8 cM between the two flanking markers B1 and B4 (Miftahudin et al. 2004) covered a 142-kb region of a 160-kb rice BAC clone (gi:18997259). The barley cDNA (BCD1230) sequence co-segregated with the *Alt3* gene and was identical with part of the ribulose phosphate epimerase (*RPE*) gene sequence located in the middle of the rice BAC (Miftahudin et al. 2004). To saturate the *Alt3* region in rye, we developed PCR-based RFLP markers, utilizing ten annotated gene sequences from the 142-kb rice BAC region surrounding the *RPE* gene (Table 1). The PCR products were used as probes to hybridize to rye genomic DNA from both parents that had been digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, and *Dra*I enzymes in order to identify polymorphisms, which were then mapped to the F_2 population.

High-resolution mapping of the Alt3 region in rye

Construction of a high-resolution map was carried out using all recombinant F_2 plants. Selected PCR-based RFLP markers that had been developed based on the rice BAC sequence were used to map the region between the two flanking markers B1 and B4. Southern hybridizations were carried out as described by Miftahudin et al. (2002).

Data analysis

All data for each marker from 1,123 F_2 plants were classified into homozygous tolerant (A), homozygous sensitive (B), and heterozygous (H) groups, and marker segregation in the population was analyzed using a chi-square test with a critical *P*-value of 0.05. Mapping and linkage analysis were performed using MAPMAKER, version 3.0, software (Landers et al. 1987). A linkage map was constructed with a LOD score of 5.0, and map distance was calculated using the Kosambi function (Kosambi 1944).

Results and discussion

Segregation of the flanking markers B1 and B4

All 1,123 F_2 plants were screened using PCR analysis for markers B1 and B4 (Fig. 2). Segregation analysis for both markers indicated three genotypes, which fit to a ratio of 1 (homozygous-tolerant form of the marker):2 (heterozygous):1 (homozygous-sensitive form of the marker). The chi-square values for markers B1 and B4 were 0.3777 ($P = 0.83$) and 0.3042 ($P = 0.86$), respectively. This segregation pattern was in agreement with the inheritance pattern of the same markers in the rye F_6 RIL population (Miftahudin et al. 2004).

The genetic distance between both markers in the small rye F_6 RIL population (Miftahudin et al. 2004) was 0.8 cM; thus, 18 of the 1,123 F_2 plants would be expected to show recombination between the two markers. However, PCR analysis of the 1,123 F_2 plants identified only 15 recombinant plants, which was only slightly less than expected. The genetic distance between both markers in the F_2 population was calculated to be 0.7 cM, based on 15 recombinant plants per 2,246 gametes in the F_2 population.

Locating the *Alt3* locus on the genetic map

To locate the *Alt3* locus on the genetic map, the 15 F_2 recombinant plants for the region spanning markers B1 and B4 were phenotyped, based on their F_3 progeny. Selfed seed from the recombinant plants was produced and used to obtain $F_{2:3}$ families. Using a root re-growth method (Somers et al. 1996), the $F_{2:3}$ families were classified as four homozygous-tolerant families, four homozygous-sensitive families, and seven families segregating for tolerance/sensitivity in a 3:1 ratio, indicating that the F_2 recombinant plants were composed of three genotypes, homozygous Al-tolerant (*Alt3 Alt3*), homozygous Al-sensitive (*alt3 alt3*), and heterozygous (*Alt3 alt3*), thus confirming that the *Alt3* gene was a single dominant gene.

All 15 recombinant plants were grouped into seven recombinant classes (Table 2). Among the 15 recombinant plants, seven plants had a recombination breakpoint between marker B1 and *Alt3*, and the rest had a breakpoint between the *Alt3* gene and marker B4, thus placing *Alt3* between markers B1 and B4.

Development of RFLP markers from the rice BAC clone

RFLP analysis of rice marker RZ891, which is part of the *RPE* gene in the rye F_2 population, showed only one

Table 1 Primer sequences developed from the rice BAC sequence used in this study

Primer names	Rice genes	Forward sequences (5′–3′)	Reverse sequences (5′–3′)	T_a[a] (°C)
B11	*b11*	TCACAGAGGCCCAATCGTTC	GTTCCCTTTTTCTCCGCTACAG	58
B25	*b25*	CGGTGACGGTGGAGGCGACAA	GGAAGCAACGACGTGGGAGACAGC	58
B26	*b26*	TAGCGGCGAGAAGATGGTGGTGTG	TCTCCTCCCCGGCTTTGATGTCC	60
B27	*b27*	GTCCCTCGCGTCCTCCTA	CCTCGACGCCCAACTCCT	60

[a] T_a Annealing temperature

Table 2 Aluminum (Al) tolerance phenotype classes of the $F_{2:3}$ families, based on root re-growth under 2 ppm of Al in minimal nutrient solution

Line number	Number of samples	Phenotype classes (T/S/H)[a]	Observed class of phenotypes		Expected class of phenotypes		χ^2	*P*-value
			T	S	T	S		
54	36	H	28	8	27.00	9.00	0.15	0.70
201	33	H	26	7	24.75	8.25	0.25	0.62
226	38	T	38	0	38.00	0.00	0.00	1.00
253	35	S	0	35	0.00	35.00	0.00	1.00
257	36	H	27	9	27.00	9.00	0.00	1.00
305	33	H	26	7	24.75	8.25	0.25	0.62
616	30	H	23	7	22.50	7.50	0.04	0.84
784	32	S	0	32	0.00	32.00	0.00	1.00
859	36	H	23	13	27.00	9.00	2.37	0.12
862	37	T	37	0	37.00	0.00	0.00	1.00
948	35	T	35	0	35.00	0.00	0.00	1.00
965	20	S	0	20	0.00	20.00	0.00	1.00
1,106	29	T	29	0	29.00	0.00	0.00	1.00
1,119	29	H	19	10	21.75	7.25	1.39	0.24
1,136	30	S	0	30	0.00	30.00	0.00	1.00

[a] *T* Al tolerant, *S* Al sensitive, *H* heterozygous

recombinant event between the marker and the *Alt3* locus, indicating that RZ891 was very tightly linked to the gene.

There were 36 annotated genes in the 160-kb rice BAC clone including putative, unknown, and hypothetical genes. A basic local alignment search tool (BLAST) analysis of ten putative genes surrounding the *RPE* gene against expressed sequence tag (EST) databases showed various levels of homology to ESTs from rice, wheat, barley, maize (*Zea mays* L.), and sorghum (*Sorghum vulgare* L.). PCR-based RFLP markers were developed from those genes by designing primers, using the same approach as that used for developing markers B1 and B4 (Miftahudin et al. 2004).

Of the ten screened markers surrounding the *RPE* gene, only three were polymorphic between the rye parents. Most of the ten markers showed very weak bands, even though a low stringency hybridization washing was used, which indicated that those markers had low homology with rye genomic DNA. Of the three polymorphic markers, two markers, B6 and B11, showed a single strong band, while the other one showed a single very weak band, which could not be mapped. Markers B6 and B11 were mapped in the rye F_2 population to evaluate the linkage between those markers and the *Alt3* gene. Marker B6, developed from a putative cytochrome P-450 gene, was separated by four genes from the *RPE* gene toward marker B4 in the rice BAC clone (Fig. 1). One of the four genes was a gene of unknown function from which the marker B11 was derived.

Delimitation of the *Alt3* gene region

The markers B1, B4, and RZ891 were remapped to the rye F_2 population to reveal genetic linkage in the *Alt3* region. Markers B6 and B11 were also integrated into the genetic map. The results showed that the distance between the B1 and B4 markers was 0.7 cM, with B6 and RZ891 located between those markers. Markers RZ891 and B6, which co-segregated with the *Alt3* gene in the small rye F_6 RIL population, were found to be flanking the *Alt3* gene with each marker 0.05 cM from the gene (Fig. 2).

Another PCR-based RFLP marker (B26) was developed from one of the four rice genes located in between the RPE and putative cytochrome P-450 genes in the rice BAC clone. Linkage analysis of the F_2 population revealed that two markers, B11 and B26, co-segregated with the *Alt3* locus (Fig. 2). This result was especially interesting, because both markers were derived from the rice BAC sequence in the region between the *RPE* and cytochrome P-450 genes. Those are the two genes from

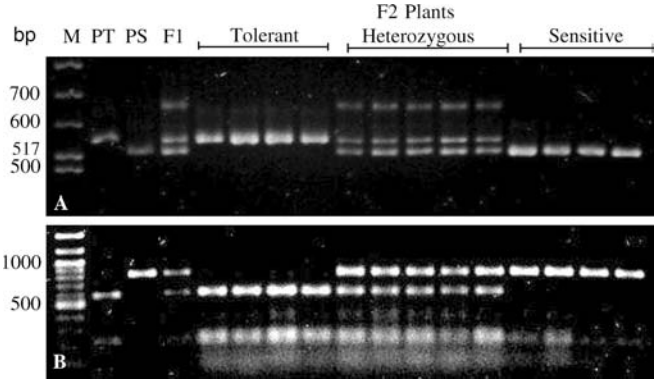

Fig. 1 Profiles of two PCR co-dominant markers in tolerant, sensitive, and heterozygous genotypes. **a** A simple co-dominant marker, B1. **b** A cleaved amplified polymorphism sequence marker, B4. *PT* Aluminum (Al)-tolerant parent, *PS* Al-sensitive parent, *F1* F_1 plant derived from a cross between PT and PS

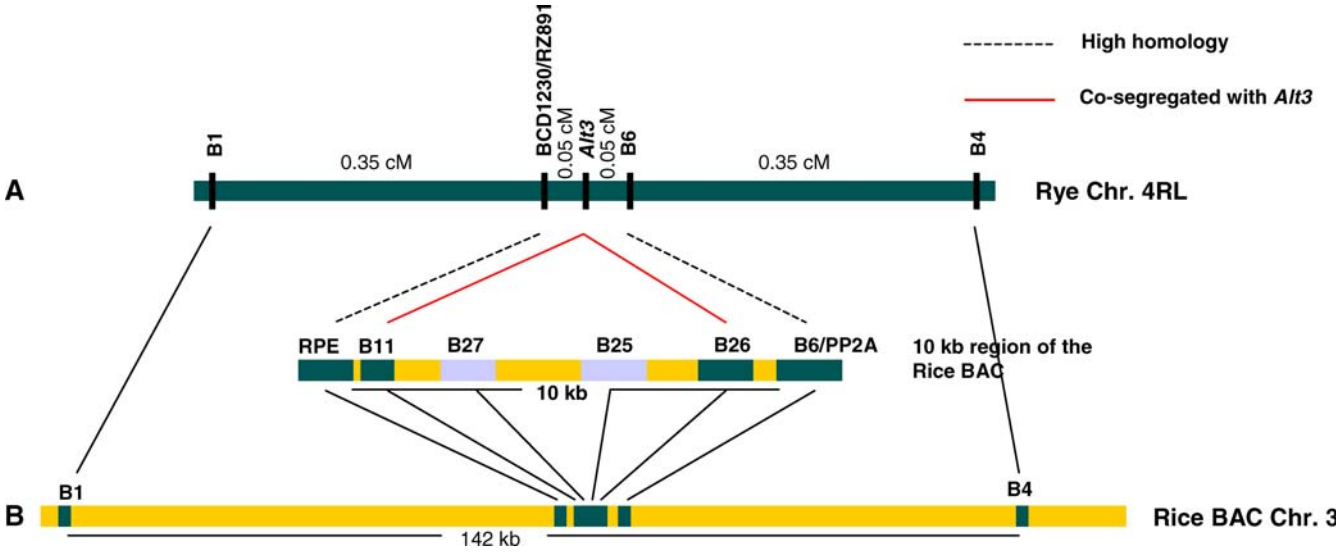

Fig. 2 Colinearity between the *Alt3* gene region and a rice BAC sequence (gi:18997259). **a** High-resolution genetic map of the *Alt3* region. All markers except RZ891/BCD1230 were developed from the rice BAC clone. The linkage map was constructed using MAPMAKER, version 3.0 (Lander et al. 1987), with a LOD value 5.0, using the Kosambi map function (Kosambi 1944). **b** Schematic diagram of the rice BAC and genes in between markers B1 and B4

which markers BCD1230/ RZ891 and B6 were derived, respectively. Since B11 is located just to the right of the *RPE* gene and B26 is just left of cytochrome P-450 genes on the rice BAC (Fig. 1), *Alt3* could be located in the B11–B26 interval, or it could be either B11 or B26. RFLP analysis of the other two markers located between B11 and B6 showed no polymorphism between the two parents; therefore, we were unable to locate those markers on the high-resolution map.

Unfortunately, primers constructed from the ends of the rice gene, B26, and from the B26 flanking regions did not work in rye. It appears that both the 3′ and 5′ ends of the rice gene, B26, and the flanking regions have changed considerably from the time rice and rye separated from their progenitor. However, PCR-based primers made from a small region in the middle of the rice B26 gene did amplify in rye and were used to map the gene in rye.

Micro-colinearity between rice and rye

Comparative mapping studies among grass species have indicated that there is a high level of gene order conservation at the macro level (Ahn et al. 1993; Kurata et al. 1994; Moore et al. 1995; Van Deynze et al. 1995a, b, c; Korzun et al. 1997; Gale and Devos 1998; Gallego et al. 1998; Han et al. 1998; Kato et al. 1999). Studies of the *Sh2*/*A1* and *adh* orthologous region in rice, sorghum, and maize showed gene colinearity at the micro level (Chen et al. 1997, 1998; Tikhonov et al. 1999; Tarachini et al. 2000; Bennetzen and Ramakrishna 2002). However, there are some exceptions where linked markers did not map to the predicted locations within colinear region (Bennetzen et al. 1998; Wilson et al. 1999; O'Neill and Bancroft 2000) or gene sequence, order and orientation varied among those species (Tikhonov et al. 1999; Tarachini et al. 2000; Bennetzen and Ramakrishna 2002). This variation in syntenic relationship complicated the usefulness of information from comparative genetics for identification and cloning genes of interest.

Attempts to clone a gene, based on comparative genetics information between rice and other grass species, were initiated when Killian et al. (1997) attempted to clone the stem rust resistance gene *Rpg1* from barley, based on homoeologous relationships between barley and rice. Although both regions exhibited good colinearity, the target gene could not be identified in the predicted location in rice. This is similar to the present attempt to characterize a rye gene, using a rice gene sequence and its flanking region as a source of primers. It appears that in some cases, as noted by Killian et al. (1997) and in the present study, the rice sequence can be a valuable source of PCR-based primers only up to a point, and then micro-sequence variation between rice and the second species prevents further progress.

The *Alt3* region between markers B1 and B4 on the rye chromosome 4RL showed very good colinearity with the rice chromosome 3 BAC sequence. All five rice markers that mapped in the rye *Alt3* region were located in the rice BAC clone in same linear order (Fig. 2). If the physical distance between B1 and B4 were less then a megabase, this result would be the first report of a microsyntenic relationship between rice and rye at the kilobase level. The *Alt3* region in the B1–B4 interval covers 142 kb with 33 annotated genes, suggesting that in rice this is a gene-rich region with a density of 4.3 kb per gene.

The *Alt3* gene region in rye

The genetics of Al tolerance have been studied in many plant species including wheat (Riede and Anderson 1996;

Rodriguez Milla and Gustafson 2001; Sasaki et al. 2004), barley (Tang et al. 2000), rye (Aniol and Gustafson 1984; Gallego et al 1998; Miftahudin et al. 2002), maize (Sibov et al. 1999), and rice (Wu et al. 2000; Nguyen et al. 2001). However, there has been no report of the construction a high-resolution map of any Al tolerance gene region that might facilitate gene cloning. We constructed a high-resolution map of the *Alt3* gene in rye for three reasons. First, our previous results showed that rye, an outcrossing species, was highly polymorphic for the region (Miftahudin et al. 2002). Since development of a genetic map is dependent on the polymorphism level of the parental species, we expected to be able to construct a high-resolution map of the *Alt3* region. Second, there appears to be a good macrosyntenic relationship between the genomes of various members of the Triticeae and rice in the Al tolerance region (Miftahudin et al. 2002). Therefore, we could employ the rice genome sequence data as a source of sequence-based markers to saturate the *Al3* region in rye. Third, since rye is the most Al-tolerant species in the Triticeae, we expected that high-resolution mapping of the *Alt3* gene in rye could facilitate the isolation, characterization, and cloning of an important gene responsible for Al tolerance.

The *Alt3* region in the long arm of rye chromosome 4 was saturated with RFLP markers developed from a rice chromosome 3 BAC clone sequence. Two co-segregating markers, B11 and B26, and two flanking markers, RZ891/ BCD1230 and B6, were mapped to the region. Although the physical distance in rye between both flanking markers has not been determined, it was expected that they would be physically close to each other, since the corresponding *Alt3* region in the rice BAC appears to be a gene-rich region. In this study, the genetic and physical relationship of the *Alt3* region is not critical, because the aim of the high-resolution map was to identify an orthologue *Alt3* gene from the rice BAC. The availability of the rice BAC sequence was more important. Fortunately, the rice BAC clone used in this study has been fully sequenced and annotated (Buell et al. 2002). Among the 33 annotated genes in the region between sucrose transporter 1 (*SUT1*) and protein phosphatase-2A (*PP2A*) genes of the rice BAC, four functional genes—sucrose transporter 1, protein phosphatase PP2A sub unit 5, *RPE*, and cytochrome P-450—showed a high homology with rye genomic DNA. The first two genes showed homology between the two species, based on the sequence comparison (Miftahudin et al. 2002), and the other two showed homology, based on a strong hybridization signal. It is noteworthy that the *ALMT1* gene characterized by Sasaki et al. (2004) did not show sequence similarity with any part of the rice BAC utilized in the present study.

Searching for a candidate *Alt3* gene

There are several techniques available to isolate a gene from plants, including the use of heterologous probes and transposon tagging. In a species such as rye, which lacks those tools, positional cloning is an alternative choice; however, rye, at the present time, does not meet the requirements for either chromosome walking or landing for two reasons. First, rye has a very large genome size, which is composed of 85% or more repetitive sequences (Arumuganathan and Earle 1991). Second, there is no large insert library available in rye. Therefore, the approach we used combined a map-based-cloning technique with knowledge of the rye/rice syntenic relationship. We utilized rice genome sequences developed from a rice BAC as a source of markers to saturate and delimit the *Alt3* region in rye.

Another possible advantage to using these techniques is that an orthologue *Alt3* gene might be identified if the regions demonstrated a good microsyntenic relationship at the kilobase level. The candidate orthologue *Alt3* gene in the rice BAC was physically delimited using flanking markers from the high-resolution map of the *Alt3* region. Our results showed that the *Alt3* region spanning the flanking markers RZ891–B6 covers a 10-kb region of the rice BAC clone, which contains one unknown and three hypothetical genes. If the syntenic relationship is conserved between the 10-kb region and the *Alt3* region in the RZ891–B6 interval, one of four genes should be a candidate orthologue *Alt3* gene and could be used to tag the *Alt3* gene in rye. BLAST search analyses of those four genes showed that two genes, including the unknown gene from which marker B11 was derived, have high homology with several ESTs from the shoot and reproductive organs of rice, maize, and sorghum, suggesting that those two genes were unlikely orthologues of the *Alt3* gene. The other two genes had no significant homology with any EST in any database.

To characterize the four coded sequences of rice that might be a candidate gene, we performed Northern blot hybridization analysis, using total RNA isolated from root tips of the Al-tolerant and -sensitive rye parents. The root tips were treated with 2 ppm of Al (Miftahudin et al. 2002) for 0-, 1-, 6-, and 12-h time courses. The results showed that none of the four rice candidate genes was the likely candidate *Alt3* orthologue gene, suggesting that major genome rearrangements (i.e., a translocation, inversion, mutation, or deletion) had occurred within the genes of this region during evolutionary separation of rice and rye.

However, the high-resolution map of the *Alt3* region in rye, using a rice BAC clone did provide a fundamental cloning tool and opened up the possibility of cloning the Al tolerance gene in rye and in other members of the Triticeae. Since it was not possible to identify and characterize the orthologue *Alt3* gene from the existing rice BAC sequence, the region of the rye genome spanning the flanking markers will need to be physically determined and cloned. This will facilitate sequencing the potential *Alt3* region between the two flanking markers.

Using primers developed from a segment of the rice BAC B26 gene, we identified a rye DNA fragment with

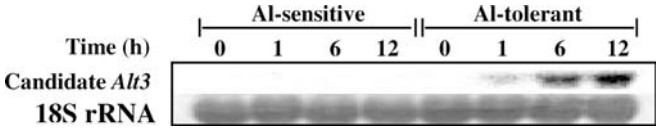

Fig. 3 Time course gene expression analysis of the rye candidate *Alt3* gene. The rye probe R26A was hybridized with the total RNA isolated from root tips of rye Al-tolerant and Al-sensitive parents, following treatments under Al stress for 0, 1, 6 and 12 h

the potential to be part of an Al-induced gene. Based on the Northern hybridization data, using this DNA fragment as a probe, we suggest that the gene could be responsible for Al tolerance in rye (Fig. 3). This gene was expressed only in root tips of the Al-tolerant parent, and only when that tolerant parent was under Al stress. This gene appears to be considerably different from the *ALMT1* gene characterized by Sasaki et al. (2004), which is constitutive in wheat and appears to be constitutively expressed in root tips of tolerant and moderately tolerant cultivars, regardless of whether or not they were stressed (see Sasaki et al. 2004; Fig. 2b). However, until today we have not been able to isolate the full rye-derived sequence. PCR walking, 5′ RACE, and cDNA-screening techniques have been used in our attempts to isolate a complete sequence of the gene; however, those techniques have not been very successful. A rye large-insert library and further analysis are needed to characterize the gene.

The flanking markers, BCD1230/RZ891, and B6, together with the co-segregating markers, derived from B11 and B26 can also be used to screen the wheat D-genome BAC library. Since BCD1230 was mapped on wheat chromosome 4DL, and cytochrome P-450 is also a common gene, it may be possible to identify a wheat D-genome BAC clone that contains both markers, which will allow for the isolation of an orthologous gene in wheat.

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