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Single nucleotide polymorphism markers linked to root elongation rate in sugar beet♣

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Abstract

The aim of this study was to identify single nucleotide polymorphism (SNP) markers genetically linked to root elongation rate (RER) in sugar beet (*Beta vulgaris* L.). A population of 244 F₃ individuals, obtained from the cross between lines L01 (low RER) and L18 (high RER), was phenotyped by measuring RER of 11-d-old seedlings grown in hydroponic culture. Two DNA bulks of 50 F₃ individuals with extremes phenotypes were used for bulk segregant analysis (BSA) by restriction-associated DNA (RAD) sequencing. A total of 20 376 SNP were identified. SNPs were filtered to reduce the number of false positive and mapped on candidate chromosomal regions the *B. vulgaris* reference genome. A total of SNPs were selected one of which, SNP10139, was strongly linked ($P < 0.01$) to RER. The pattern of association between SNP10139 genotype and RER was also evaluated on a breeding line panel comprising 40 low and 40 high RER individuals with different allele frequencies between groups ($P < 0.01$). The SNP10139 sequence was mapped the *B. vulgaris* peptide transporter (*PTR*) gene, a carrier that influences root elongation in *Arabidopsis thaliana*. Our results suggest that SNP10139 influences RER in sugar beet and sequence information can be used in marker-assisted selection programs.

Additional key words: abiotic stresses, *Beta vulgaris*, bulk segregant analysis, restriction-associated DNA sequencing.

Introduction

Roots play a central role in water and nutrient acquisition and root characteristics involved in these functions are closely associated with crop productivity (Lynch *et al.* 1995). The ability of a plant to absorb nutrients distributed in the soil is given by the morphology of its root system. The improvement of root characteristics is essential to increase crop yield especially in environments subjected to recurrent water and nutritional stresses (De Dorlodot *et al.* 2007).

Lynch (2013) proposed a maize ideotype, termed “Steep, Cheap, and Deep”, for superior nutrient and water acquisition. Root traits influencing rapid soil exploitation, such as root elongation rate (RER), could be used to develop crops with greater water and nutrient acquisition (Lynch *et al.* 2014, Saengwilai *et al.* 2014). Recent study of sugar beet highlighted that key root traits as RER, total length, surface area, and number of tips are strictly related to sulfate acquisition and sugar beet yield

(Stevanato *et al.* 2010). A significant and positive correlation was demonstrated between yield and nitrogen uptake rate in sorghum or sulfate uptake rate in maize and sugar beet (Cacco *et al.* 1980, Saccomani *et al.* 1981, Nakamura *et al.* 2002, Stevanato *et al.* 2014).

The improvement of root traits through conventional breeding methods is slow because they are controlled by multiple gene loci (De Dorlodot *et al.* 2007), but with the assistance of molecular markers faster genetic control and improvement of plant root apparatus can be achieved. Marker-assisted selection allow determine major loci controlling root traits in rice (Courtois *et al.* 2003) and soybean (Liang *et al.* 2010). Root morphology is controlled by many genes that interact with the environment and were found to explain up to 30 % of phenotypic variation (Price *et al.* 2002, Giuliani *et al.* 2005). Similarly, Tuberosa *et al.* (2002) identified quantitative trait loci (QTLs) influencing root architecture

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Abbreviations: BSA - bulk segregant analysis, RAD - restriction-associated DNA sequencing, *PTR* - peptide transporter gene; RER - root elongation rate; SNP - single nucleotide polymorphism.

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and yield in maize. Single nucleotide polymorphism (SNP) markers have gained popularity in crop breeding programs by increasing the efficiency and accuracy of selection procedures (Ganal *et al.* 2009). SNP are ideal markers for identifying genes associated with useful traits in crops because they are abundant and densely located on plant genomes. The application of next-generation sequencing technology greatly facilitated high throughput SNP discovery, and a large number of commercial platforms are available for automated SNP genotyping (Gupta *et al.* 2008).

Bulk segregant analysis (BSA) is a method for identifying DNA markers linked to genes or genomic

regions of interest (Michelmore *et al.* 1991). DNA samples from individuals showing contrasting phenotype are compared with a large set of molecular markers to identify linkage to the trait of interest. This procedure has been successful in the detection of major genes implicated in lateral root growth in rice (Wang *et al.* 2006) or root development in response to aluminum stress in barley (Raman *et al.* 2002) and wheat (Cai *et al.* 2008).

The objective of this study was to identify SNP markers linked to RER in sugar beet by means of BSA and to map SNP sequences to the reference *B. vulgaris* genome to identify candidate genes influencing root elongation.

Materials and methods

Plants: A population of 244 F₃ individuals, obtained from the cross between lines L01 (low RER; 1.7 mm d⁻¹) and L18 (high RER; 20.5 mm d⁻¹), was phenotyped by measuring RER of 11-d-old seedlings grown in hydroponic culture. The F₃ plants were derived from a single F₁ individual and by single-seed descent of 244 F₂ plants grown at the University of Padova (Italy). The pattern of association between genotypes and RER was also evaluated on 80 individuals of a F₂ population, named F290, showing a wide variation for RER and provided by *Lion Seeds* (Maldon, UK).

Root elongation rate analysis: Seeds were surface-sterilized by immersion in 1 % (m/v) sodium hypochlorite, rinsed several times with distilled water, then imbibed in aerated, deionized water at 22 °C for 12 h. Seeds germinated between two layers of filter paper moistened with distilled water in Petri dishes at 25 °C in the dark for 48 h. Only 3-d-old seedlings with 10 ± 2 mm long seminal roots were transferred into hydroponic plastic tanks with an aerated nutrient solution containing 200 µM Ca(NO₃)₂, 200 µM KNO₃, 200 µM MgSO₄, 40 µM KH₂PO₄, and microelements (Arnon and Hoagland 1940). Nutrient solution was replaced daily. Tanks were placed in a growth chamber set at day/night temperatures of 25/18 °C, relative humidity of 70/90 %, a 14-h photoperiod, and an irradiance of 60 W m⁻². Primary root length of individual seedlings was manually measured each day until seedlings were 11-d-old. The RER was calculated as the difference in root length between two measurements with *WINRHIZO Pro* software (*Regent Instruments*, Quebec, Canada). Trait distribution was tested for normality with Shapiro-Wilk test (Conover 1980).

SNP discovery by RAD-BSA: DNA was isolated from 20 mg of leaf tissue with *BioSprint 96* DNA plant kit in a *BioSprint 96* workstation (*Qiagen*, Germany) following the manufacturer's instructions. DNA was assayed for concentration and purity by microfluidic gel electrophoresis with *Agilent 2200 TapeStation* system (*Agilent Technologies*, CA, USA). Based on the F₃ samples

phenotyping analysis, DNA of the 50 individuals with extreme low and high RER were selected for BSA restriction-associated DNA (RAD) analysis (*FloraGenex*, OR, USA) following the methods outlined by Pegadaraju *et al.* 2013. Briefly, 100 bp paired-end *Illumina* sequences were obtained from the bulks. Restriction enzyme-derived reads were first trimmed to remove low quality sequences with an average phred-scaled quality score below 25 (Q25) and then collapsed into RAD clusters sharing complete sequence identity across the sequence flanking the restriction site. Only sequences with coverage between 20× and 1000× were considered in the analysis. The paired-end sequences were extracted for each RAD cluster passed to the *Velvet* sequence assembler for contig assembly and then aligned using *Bowtie*, allowing up to 3 base pair mismatches between the paired-end read and the reference. Sequence variants were then followed using *SAM* tools. To provide a genomic anchor and location for the newly discovered SNP, the RAD cluster sequences were aligned and mapped on the sugar beet reference genome (v. *RefBeet-1.1*; <http://bvseq.molgen.mpg.de>) using *BLASTN* (v.2.2.27) and allowing for a maximum of a single mismatch. For putative gene identification some selected sequences were analyzed against the *Arabidopsis* genome database (*TAIR v. 10*) *TBLASTX*, using a maximum threshold E-value of 10⁻¹⁰ (Altschul *et al.* 2010).

Linked-SNP validation by genotyping: From the SNP discovery analysis, a total of candidate SNPs were selected for validation on the 100 F₃ samples with extreme phenotypes used for the BSA and on 80 F₂ samples with different RER from a breeding population. SNP genotyping was performed using the *QuantStudio 12K Flex* real-time PCR system and *OpenArray* technology (*Life Technologies*, CA, USA) following the manufacturer's instructions. Briefly, 10 ng of DNA was mixed with 2.5 mm³ of *TaqMan OpenArray Genotyping Master* and amplified. Results were analyzed using the *Taqman Genotyper* software (v.1.0.1) and χ^2 -test was adopted for the association analysis between phenotypic and genotypic data.

Results

Root elongation rate (RER), evaluated on 244 individuals of the F_3 progeny, showed a normal distribution ($W = 0.979$; $P < 0.01$) with a certain degree of transgressive segregation for high values of RER (Fig. 1).

Bulks were obtained from 50 F_3 individuals with most extreme phenotypes; low and high RER bulks were characterized by average RER of 6.3 ± 1.3 and 40.0 ± 8.9 mm d⁻¹, respectively (Fig. 1).

Fig. 1. Frequency distribution of root elongation rate in 244 F_3 individuals from L01 $\times$ L18.

Table 1. Frequency of the number of SNPs identified per sequence. Total number of sequences = 14 459.

Number of SNP per sequence	Number of sequence	Percentage
1	9872	68.28
2	3403	23.53
3	1056	7.30
4	111	0.77
5	16	0.11
6	1	0.01

Illumina RAD sequencing yielded 68 360 881 raw reads of high quality with an average length of 102.4 nt. Overall, a total of 20 376 SNP were identified on 14 459 alignments (Table 1). Most of the sequences presented a single SNP, while the remaining showed two or more SNPs in the same sequence. The majority of SNPs were diallelic (98.8 %) and more transitions (12 378) than transversions (7 746) were observed (1.6 ratio).

In order to reduce false positive SNP association, appropriate quality filters were adopted; only sequences harboring single and diallelic SNP were selected and then aligned to the sugar beet reference genome. For the association between allele frequencies and RER phenotypes only SNP with similar sequence coverage between bulks were selected (< 20 % coverage difference). Allele frequency ratio between bulks was estimated and only ratios higher than 2 (or lower than

0.5) were considered as candidate SNPs linked to the RER trait. A total of 234 SNPs passed the quality and association criteria and were selected as candidate markers associated to the RER trait.

The selected 234 SNPs were distributed across all 9 sugar beet chromosomes and located on 78 scaffolds (Table 2). SNPs sequences and their corresponding mapping coordinates are reported as Table 1 Suppl. The highest number of SNPs was observed in chromosome 8 (56) and the lowest in chromosome 7 (4). Among scaffolds with multiple mapped SNPs, the Bvchr8.sca002 on chromosome 8 showed the highest number (13).

SNPs were genotyped on the DNA of the individuals from the extremes of the phenotypic distribution for

Table 2. Distribution of the 234 selected SNPs putatively linked to root elongation rate on the sugar beet genome (*RefBeet-1.1*).

Chromosome	Size [Mb]	Number of SNPs	Number of scaffolds
1	41.5	20	6
2	39.5	40	16
3	32.3	18	9
4	31.1	23	9
5	56.2	25	11
6	57.8	41	11
7	50.9	4	1
8	40.1	56	12
9	45.2	7	3

validation. The significant association was found for SNP10139 ($P < 0.01$). Sequences of the primers and *TaqMan* probes designed for the detection of SNP10139 are reported (Table 2 Suppl.). SNP10138 was the only SNP located on the same scaffold (Bvchr6.sca002) of SNP10139. Nevertheless, SNP10138 was statistically not associated to the RER trait ($P < 0.07$). The pattern of association between genotypes of SNP10139 and RER was also evaluated on 40 low and 40 high RER individuals of the F_2 population F290, showing a wide variation for RER trait. Low and high RER samples were characterized by an average RER of 1.2 ± 0.02 and 2.8 ± 0.08 mm d⁻¹, respectively (Table 3). For this breeding population SNP10139 also showed different

allele frequencies between the two groups ($P < 0.01$).

On the sugar beet reference genome the SNP10139 was mapped on chromosome 6 within the fourth exon of the *Bv6_128350_ktfi* gene (Fig. 2). Interestingly, *Bv6_128350_ktfi* sequence showed a high homology with the peptide transporter gene (*PTR*) family of *Arabidopsis thaliana* (Fig. 3). Among this family, *AtPTR2* (AT2G02040.1) showed the highest similarity (72.8 %) with the *Bv6_128350_ktfi* gene (Table 4). However, the SNP10139 polymorphism is characterized by a mutation from A to G on the third base of the codon for leucine (UUA > UUG), which results in a silent mutation.

Table 3. SNP10139 alleles in low and high root elongation rate (RER) samples in the F290 F_2 population.

Low RER samples			High RER samples		
Sample ID	RER [mm d ⁻¹]	SNP10139 genotype	Sample ID	RER [mm d ⁻¹]	SNP10139 genotype
L1	0.9	A/A	H1	2.4	G/A
L2	0.9	A/A	H2	2.4	A/A
L3	1.0	A/A	H3	2.4	A/A
L4	1.0	A/A	H4	2.4	G/G
L5	1.0	A/A	H5	2.4	A/A
L6	1.0	A/A	H6	2.5	G/G
L7	1.1	G/G	H7	2.5	G/G
L8	1.1	G/G	H8	2.5	G/A
L9	1.1	G/G	H9	2.5	G/G
L10	1.1	G/G	H10	2.5	A/A
L11	1.1	A/A	H11	2.6	G/G
L12	1.1	A/A	H12	2.6	G/G
L13	1.1	A/A	H13	2.6	G/A
L14	1.1	A/A	H14	2.6	G/A
L15	1.1	A/A	H15	2.6	A/A
L16	1.1	A/A	H16	2.6	A/A
L17	1.1	A/A	H17	2.7	G/G
L18	1.1	A/A	H18	2.7	G/G
L19	1.2	G/G	H19	2.7	A/A
L20	1.2	G/G	H20	2.7	A/A
L21	1.2	G/A	H21	2.7	G/G
L22	1.2	G/A	H22	2.7	G/A
L23	1.2	A/A	H23	2.7	G/A
L24	1.2	A/A	H24	2.7	A/A
L25	1.2	A/A	H25	2.7	A/A
L26	1.2	A/A	H26	2.7	A/A
L27	1.3	G/G	H27	2.7	A/A
L28	1.3	G/G	H28	2.8	G/G
L29	1.3	A/A	H29	2.8	G/G
L30	1.3	A/A	H30	2.8	G/A
L31	1.3	A/A	H31	2.8	G/A
L32	1.3	A/A	H32	2.8	A/A
L33	1.3	A/A	H33	2.9	A/A
L34	1.3	A/A	H34	2.9	A/A
L35	1.3	A/A	H35	3.1	G/G
L36	1.4	G/A	H36	3.2	A/A
L37	1.4	A/A	H37	3.4	G/A
L38	1.4	A/A	H38	3.8	G/G
L39	1.5	A/A	H39	3.8	G/G
L40	1.5	A/A	H40	5.1	G/A

Table 4. Amino acid sequence identities [%] among the peptide transporter genes *PTR* of *Beta vulgaris* (*Bv*) and *Arabidopsis thaliana* (*At*).

Species	Gene	No.	2	3	4	5	6	7
<i>Bv</i>	<i>Bv6_128350_ktfi</i>	1	72.8	62.0	56.1	60.4	59.1	41.8
<i>At</i>	<i>AtPTR2_AT2G02040.1</i>	2	-	68.0	62.7	59.1	57.1	40.2
<i>At</i>	<i>AtPTR6_AT1G62200.1</i>	3		-	63.1	53.2	52.8	37.8
<i>At</i>	<i>AtPTR4_AT2G02020.2</i>	4			-	47.2	45.9	35.9
<i>At</i>	<i>AtPTR1_AT3G54140.1</i>	5				-	73.9	42.3
<i>At</i>	<i>AtPTR5_AT5G01180.1</i>	6					-	41.6
<i>At</i>	<i>AtPTR3_AT5G46050.1</i>	7						-

Fig. 2. Distribution of selected 234 SNPs across all 9 sugar beet chromosomes (dark bars). The red bar shows the position of the SNP10139 on the chromosome 6.

Fig. 3. Schematic representation of the *Bv6_128350_ktfi* gene with the position of the SNP10139 according to the reference genome (*RefBeet-1.1*; <http://bvseq.molgen.mpg.de>).

Discussion

In this study, we demonstrated the feasibility of combining BSA and RAD approaches to generate a large number of candidate SNPs for association studies with RER in a format suitable for high-throughput genotyping. Our approach provides a good example of the high potential of RAD technology, combined with comparative assembly to the sugar beet genome, to characterize large numbers of informative SNPs in pooled DNA samples. Analogous approaches were successfully used to identify a panel of SNPs in eggplant (Barchi *et al.* 2011) and sugar beet (Stevanato *et al.* 2014).

Among associations between SNP mutations and the RER trait in sugar beet, we identified a strong association for SNP10139. Analogously, Rosas *et al.* (2013) found a SNP influencing root system architecture on two candidate genes (*RSAL* and *PHO1*) of *Arabidopsis thaliana* and Kumar *et al.* (2014) revealed several SNP polymorphisms, within the *Rtcl*, *Rth3*, *Rum1*, and *Rull* genes, associated with seedling root traits in maize. The homologue peptide transporter gene (*PTR*) of *Bv6_128350_ktfi*, where SNP10139 was mapped,

influences not only root development but also the uptake of nitrate and peptides from the soil in *Arabidopsis thaliana* (Bai *et al.* 2013). Similarly, Komarova *et al.* (2008) showed that over-expression of a dipeptides transporter *AtPTR5* could enhance root growth and increase N content. Fan *et al.* (2014) demonstrated in rice that the di/tripeptide transporter *OsPTR6* increases both growth and N accumulation. These results could help to explain the previously found close association between the morphological and related physiological root traits and productivity in sugar beet (Stevanato *et al.* 2010).

Intriguingly the SNP10139 polymorphism is a silent mutation that does not result in amino acid change. A biological explanation for the effect of this SNP could be that it may be in linkage disequilibrium with another mutation in coding regions resulting in the modification of the expression of the gene. Alternatively, this SNP might change the substrate specificity of the RNA influencing the timing of translation and protein expression (Kimchi-Sarfaty *et al.* 2007). Numerous examples have been reported in the literature for linkages

between silent mutations and phenotype alterations (Goymer 2007, Garg *et al.* 2012, Jha *et al.* 2015). An association between root morphology and synonymous SNPs was recently found in maize (Abdel-Ghani *et al.* 2015) and rice (Li *et al.* 2015).

Previous studies have demonstrated that differences in gene expression can be associated with quantitative traits and SNPs. Jaiswal *et al.* (2015) found a SNP modulating the expression of the gene *TaGW2* associated with grain mass in wheat. Further studies will investigate both the functional effect of SNP10139 alleles on *PTR* gene expression and root morphology in sugar beet.

Root breeding has been proposed as a key factor for the “second green revolution” (Lynch 2007). Nevertheless, the contribution of sugar beet root traits as tools for the selection of high yielding cultivars has not been adequately taken into account in breeding programs. The molecular marker associated with root growth identified

here is one of the most efficient ways for improving root apparatus in sugar beet. The introgression of the SNP10139 allele into sugar beet genotypes might improve root soil exploration and nutrient acquisition. Previous studies in maize and sugar beet showed that rapid primary root growth plays a major role in nutrient uptake and productivity and it was hypothesized that alleles promoting root growth may facilitate selection for efficient nutrient use (Stevanato *et al.* 2010, Vamerali *et al.* 2003).

In conclusion, this study provides further indication of the polygenic control of root elongation rate in sugar beet. Moreover, our results suggest that the use of SNP10139 marker in gene-assisted selection programs offers an opportunity to improve sugar beet root development and nutrient acquisition, facilitating the selection of high yielding cultivars.

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