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Characterization of two poplar homologs of the *GRAS/SCL* gene, which encodes a transcription factor putatively associated with salt tolerance

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Soil salinity is one of the most severe abiotic stress in the Vojvodina region (Serbia), and efforts are being undertaken to enhance the salt tolerance of economically important poplar clones. We screened nucleotide diversity in candidate genes (CG) in several poplar clones to search for associations with salt stress tolerance. As plant-specific *GRAS/SCL* transcription factors (TFs) play a relevant role in abiotic stress resistance, two poplar homologs of *GRAS/SCL* TFs were chosen to differentiate the species background with respect to salt tolerance. A BLAST search of the *Populus trichocarpa* genome using the *P. euphratica* gene *GRAS/SCL TF_GH611858* sequence allowed the identification of two putative orthologs on *Scaf_5* and *Scaf_7*, with identities of 100% and 94%, respectively. Primers were designed in shared, identical sequences of *Scaf_5* and *Scaf_7* to amplify fragments of *GRAS/SCL TF* orthologs in four salt-tolerant poplar clones economically important to Serbia. The primers spanned regions where single nucleotide polymorphisms (SNPs) were present in the *P. trichocarpa* gene, thereby increasing the probability of distinguishing *Scaf_5* and *Scaf_7* orthologs in the four clones. Alignments and analyses of the gene fragments revealed that both orthologs were representative of the genetic diversity between different poplar clones, and the identified SNP markers differentiated the four poplar clones with respect to salt tolerance.

Keywords: Candidate Gene, Nucleotide Polymorphism, Tree Genomics, Poplar

Introduction

Populus deltoides Marsh. (cottonwood) of the section *Aigeiros* is one of the most important species for interspecific poplar breeding programs worldwide (Zsuffa 1975, Rahman & Rajora 2002). Clones of *P. deltoides* species (B229, PE19/66, 182/81) and *P.* × *euramericana* hybrid clone (M1) belonging to the *Aigeiros* section are of high economic importance and widely used in the forest sector in Serbia. These clones have long been cultivated in nurseries and plantations of the Institute of Lowland Forestry and Environment (ILFE) in Novi Sad, Serbia. Until now, four out of the 16 new poplar cultivars registered at the ILFE have been introduced into newly established plantations. So far, Serbian forest breeding programs at ILFE are based on a conventional clonal identification system, which relies on morphological and phenological characterizations (Orlovic et al. 1997).

Molecular markers such as AFLPs (dominant amplified fragment length polymorphisms) and SSRs (highly polymorphic, consistent and co-dominant simple sequence repeats), allow to obtain unique genetic profiles of individuals and are therefore excellent tools for clone identification (Dayanandan et al. 1998, Rahman et al. 2000, Van der Schoot et al. 2000, Rajora & Rahman 2003). Using these markers, newly selected *Populus deltoides* and *P.* × *euramericana* hybrid clones were genetically differentiated, and their genetic relationships within the population and with the most relevant clones were obtained (Galovic et al. 2004, Galovic & Orlovic 2007, Orlovic et al. 2009).

Plant productivity is greatly affected by various environmental stresses. Soil salinity is one of the most severe abiotic stress factors, causing increasing agricultural and environmental problems worldwide (Chen & Polle 2010). According to Ivanišević et al. (2008), salt-affected soils currently comprise about 5.5% of the arable land in the Vojvodina region (Serbia). Moreover, soil salinization in Vojvodina may be further increased in the next years as a consequence of the climate change. This calls for a deeper knowledge of the genetic mechanisms underlying the response of forest species to abiotic stress.

In the past decades, a number of genes encoding different structural proteins have been studied with the aim of developing a range of abiotic stress-tolerant plants. Currently, the scientific community is focusing on the use of regulatory genes, such as transcription factors (TFs), as a more effective approach for the development of stress-tolerant plants (Sairam & Tyagi 2004). According to Gu et al. (2004), the identification of a core set of stress-related transcripts by functional genomics studies are crucial for the screening of tolerant germplasms aimed at crop improvement.

Although numerous genetic studies on the adaptability of forest tree species have been published, little is known on the molecular basis of the adaptation process (Fladung & Buschbom 2009). Nucleotide diversity was screened in several candidate genes (CGs) putatively correlated with abiotic stress responses in several poplar clones. Plant-specific *GRAS/SCL* TFs are known to play diverse roles in abiotic stress resistance. According to Bolle (2004), *GRAS* proteins are

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Tab. 1 - General information on the four salt-tolerant poplar clones investigated in this study.

No	Clone collection No.	Species
1	B229	*P. deltoides*
2	PE19/66	*P. deltoides*
3	M1	*P.* × *euramericana*
4	182/81	*P. deltoides*

Tab. 2 - List of primer sequences and primer combinations used in this study.

Homolog	Sequence number	Primer sequence	Primer combinations
Scaf_7	F833	5'-CAC CAC CAT AAC CAA CAG CAA-3'	-
	R835	5'-GTG TAC ATC CCA AAA TCA GTA C-3'	F833/R835
	F836	5'-GTA CTG ATT TTG GGA TGT ACA C-3'	F836/R838
	R838	5'-AGT TGC CTC AAG AAT TGC TTG-3'	F839/R840
	F839	5'-CAA GCA ATT CTT GAG GCA ACT-3'	-
	R840	5'-CAA ATC ACT GTA ATT GCA ATA CC-3'	-
Scaf_5	F854	5'-ACC ACC CCC AGC AAC AAT TT-3'	-
	R855	5'-CGG TAA ATG CCG AAA-3'	F854/R855
	F856	5'-ATG CTG ATT TCG GCA TTT ACG G-3'	F856/R857
	R857	5'-GGT CGC TTC AAG AAT TGC TTG-3'	F858/R859
	F858	5'-CAA GCA ATT CTT GAA GCG ACC-3'	-
	R859	5'-CAT AGT ACT GGA ATT GTA ATT CC-3'	-

unique to plants, and *GRAS* homologs have been found in many higher plant species other than *Arabidopsis*. Although the *Arabidopsis* genome encodes at least 33 *GRAS* protein family members, only a few have been characterized in other plant species so far. In addition to their possible role in abiotic stress resistance, *GRAS* proteins also seem to play an important role in signal transduction, meristem maintenance and development.

Physh et al. (1999) identified a number of *Arabidopsis* expressed sequence tags (ESTs) showing high similarity to the *Arabidopsis SCARECROW* (*SCR*) amino acid sequence, thereafter designated as *SCARECROW-LIKE* genes *(SCL)*. Recently, Ma et al. (2010) showed that the *SCL* poplar gene *PeSCL7* is induced by drought and high salt stress, and encodes a member of the stress-responsive *GRAS/SCL* transcription factors (TFs). They concluded that this gene is potentially useful for engineering drought and salt tolerance in trees.

The aim of this paper was to identify homologs of the *P. euphratica* gene *GRAS/SCL TF_GH611858* in the *P. trichocarpa* genome, and to estimate their nucleotide diversity in different salt-tolerant poplar clones, as well as any possible association with salt tolerance.

Tab. 3 - Nucleotide polymorphisms detected in the gene *Scaf_7*. The IUPAC code specification for DNA sequences is: (Y): C or T; (R) A or G; (W): A or T; (K): G or T; (S): G or C; (M): A or C. (1): Base position in the sequence beginning from the start codon of *P. trichocarpa*; (2): nucleotide observed at that position in the clone sequence; (3): nucleotide at the same position in the *P. trichocarpa* gene.

***Populus* clone and primer pair used**	**Base position (1)**	**Observed Nucleotide (2)**	**Reference Nucleotide (3)**	**Type**
3-833 and 3-835	475	Y	T	Heterozygous
1-836 and 1-838	831	Y	T	Heterozygous
2-836 and 2-838	831	Y	T	Heterozygous
3-836 and 3-838	831	Y	T	Heterozygous
4-836	831	Y	T	Heterozygous
3-836 and 3-838	841	R	A	Heterozygous
3-836 and 3-838	861	W	T	Heterozygous
1-836 and 1-838	861	A	T	SNP
2-836 and 2-838	861	A	T	SNP
4-836	861	A	T	SNP
3-836 and 3-838	917	Y	T	Heterozygous
1-836 and 1-838	917	C	T	Not specific
2-836 and 2-838	917	C	T	Not specific
4-836	918	C	T	Not specific
3-836 and 3-838	986	K	G	Heterozygous
3-836 and 2-839	1005	K	T	Heterozygous
1-836 and 1-838	1005	T	G	Not specific
2-836 and 2-838	1005	T	G	Not specific
4-836	1005	T	G	Not specific
3-836 and 3-838	1006	M	T	Heterozygous
1-836 and 1-838	1006	A	C	Not specific
2-836 and 2-838	1006	A	C	Not specific
4-836	1006	A	C	Not specific
3-836 and 3-838	1012	S	C	Heterozygous
3-836 and 3-838	1017	Y	T	Heterozygous
1-836 and 1-838	1017	C	T	Not specific
2-836 and 2-838	1017	C	T	Not specific
4-836	1017	C	T	Not specific
3-836 and 3-838	1120	Y	T	Heterozygous
1-839	1533	C	T	Not specific
2-839 and 2-840	1533	C	T	Not specific
3-839 and 3-840	1533	Y	T	Heterozygous
4-839 and 4-840	1533	C	T	Not specific

Materials and methods

Plant material, DNA extraction and molecular biology analyses

Four different poplar clones (three *P. deltoides* and one *P.* × *euramericana* - Tab. 1) were analyzed in this study. Frozen leaf material was ground under liquid nitrogen using the Retsch Bead Mill (Retsch, Duesseldorf, Germany). Genomic DNA extraction was performed using the Plant DNeasy® Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed using annealing temperatures specific for each primer combinations. PCR-amplified products were purified, analysed by agarose gel electrophoresis and sequenced. The sequence of primer combinations used in the PCRs is shown in Tab. 2.

All purified fragments were sequenced in both directions and analysed using the software package SeqMan® (DNAStar Lasergene, Madison, WI, USA). DNA polymorphism patterns were recorded across all specimens within species, and the number of polymorphic sites was obtained. Nucleotide pairwise comparisons among clone sequences allowed to obtain three different polymorphism patterns: (i) those based on the number of fixed nucleotide substitution positions in all clones; (ii) those based on the occurrence of a polymorphic pattern position in one clone but not in others; and (iii) those polymorphic positions shared by all clones.

Candidate gene selection and primer design

The selected candidate gene, *GRAS/SCL*, encodes a TF with relevance to drought and salt tolerance, as demonstrated for poplar and other organisms (Bolle et al. 2000, Bolle 2004, Ma et al. 2010). The complete sequence of the *GRAS/SCL* gene of *P. euphratica* (Accession No. GH611858) was compared to the *P. trichocarpa* genome by BLAST analysis (http://www.phytozome.net/poplar.

php - Tuskan et al. 2006), and two putative homolog sequences were obtained. Exon/intron boundaries were defined through comparisons of genomic and cDNA sequences. Primers were designed according to the available sequences, and three primer combinations for each homolog were applied to cover all the exons sequences, with amplification products between 200 and 600 bp. In total, 48 fragments were sequenced in the four *Populus* clones investigated. The amplified sequences span only the exon region. The *P. trichocarpa* sequence was taken as reference in all sequence alignments.

Results

After BLAST search of the *P. trichocarpa* genome using the *P. euphratica* gene *GRAS/SCL TF_GH611858*, two putative orthologous genes were found, *Scaf_7* and *Scaf_5*, with identities of 100% and 94%, respectively. *Scaf_7* is 1918 base pairs (bp) long, and the transcriptional start position is 1064 bp downstream with respect to that of *P. euphratica GRAS/SCL TF_GH611858*. *Scaf_5* is 1780 bp long, and the transcriptional start position is 934 bp downstream with respect to that of *P. euphratica GRAS/SCL TF_GH611858*. As already indicated by the sequence identities following the BLAST analysis, *Scaf_7* was more similar to the *P. euphratica* gene than *Scaf_5*. Neither *Scaf_7* nor *Scaf_5* did reveal any indels or microsatellites. Primers were designed for regions shared by *Scaf_5* and *Scaf_7* (*e.g.*, with identical sequences in both genes) to amplify fragments of *GRAS/ SCL TF* orthologs in the four poplar clones. However, the primers spanned regions where single nucleotide polymorphisms (SNPs) were present, at least in *P. trichocarpa*, thereby increasing the probability of distinguishing both orthologs in the four clones included in this study.

The alignment of all sequences of the gene fragments for both loci (*Scaf_5* and *Scaf_7*) allowed to obtain a picture of the genetic diversity between the four poplar clones. Three fragments of different length were obtained for both loci. The sequences amplified from *Scaf_7* were 400-540 bp, 740-1170 bp and 1350-1900 bp long, while the length of sequences from *Scaf_5* were 150-390 bp, 520-860 bp and 1000-1530 bp. Those sequences covering exons regions in both loci were amplified and analysed (Tab. 3, Tab. 4, Fig. 1, Fig. 2). The number of nucleotide polymorphisms varied between each locus. For *Scaf_7*, 12 polymorphic positions were detected, while 19 positions were found for *Scaf_5*, each revealing a different degree of polymorphism. Out of the 12 polymorphic positions in *Scaf_7*, six were heterozygous, while the other six polymorphisms were non-specific. An A-T SNP was found at the 861 base position (starting from the codon of the *P. trichocarpa Scaf_7* gene) in clones B229 and PE19/66 and 182/81, while a heterozygous polymorphism was found at the same base position in clone M1.

Tab. 4 - Nucleotide polymorphisms detected in the gene *Scaf_5*. The IUPAC code specification for DNA sequences is: (Y): C or T; (R): A or G; (W): A or T; (K): G or T; (S): G or C; (M): A or C. (1): Base position in the sequence beginning from the start codon of *P. trichocarpa*; (2): nucleotide observed at that position in the clone sequence; (3): nucleotide at the same position in the *P. trichocarpa* gene.

Clone sequences	Base position (1)	Observed Nucleotide (2)	Reference Nucleotide (3)	Type
3-854 and 3-855	184	Y	T	Heterozygous
1-854 and 1-855	287	Y	C	Heterozygous
2-854 and 2-855	287	Y	C	Heterozygous
3-856	600	S	C	Heterozygous
4-856	600	S	C	Heterozygous
3-856	796	W	A	Heterozygous
4-856	796	W	A	Heterozygous
3-856	797	M	C	Heterozygous
4-856	797	M	C	Heterozygous
3-856	814	K	G	Heterozygous
1-858 and 1-859	1003	C	T	Not specific
2-858	1003	C	T	Not specific
3-858	1003	C	T	Not specific
4-858	1003	C	T	Not specific
1-858 and 1-859	1009	A	C	Not specific
2-858	1009	A	C	Not specific
3-858	1009	A	C	Not specific
4-858	1009	A	C	Not specific
2-858	1049	S	G	Heterozygous
3-858	1049	S	G	Heterozygous
4-858	1049	S	G	Heterozygous
2-858	1050	Y	C	Heterozygous
3-858	1050	Y	C	Heterozygous
4-858	1050	Y	C	Heterozygous
1-858 and 1-859	1054	A	G	Not specific
2-858	1054	A	G	Not specific
3-858	1054	A	G	Not specific
4-858	1054	A	G	Not specific
1-858 and 1-859	1165	Y	T	Heterozygous
2-858	1165	Y	T	Heterozygous
3-858	1165	Y	T	Heterozygous
1-858 and 1-859	1267	Y	T	Heterozygous
2-858	1267	Y	T	Heterozygous
1-858 and 1-859	1286	C	T	Not specific
2-858	1286	C	T	Not specific
3-858	1286	C	T	Not specific
4-858	1286	C	T	Not specific
3-858	1303	K	T	Heterozygous
1-858 and 1-859	1327	Y	C	Heterozygous
2-858	1327	Y	C	Heterozygous
4-858	1327	Y	C	Heterozygous
1-858 and 1-859	1333	R	A	Heterozygous
2-858	1333	R	A	Heterozygous
3-858	1390	Y	T	Heterozygous
3-858	1486	M	A	Heterozygous

At the *Scaf_5* locus, a different polymorphic pattern was found, as all the clones exhibited heterozygous positions and no SNPs were found. Out of 19 positions, 15 were heterozygous and four were non-specific polymorphisms.

Homologous regions in the four different *Populus* clones were sequenced and consensus sequences were designated for each clone. Out of 33 polymorphic positions of *Scaf_7* locus, 15 were heterozygous (5%) and the majority (3.3%) belonged to the hybrid M1 clone. Most of the mutations were synonymous or silent. In sequences from *P. deltoides* clones, non-synonymous SNPs could be identified with a change of an amino acid leading to a possible effect on the protein structure. In the first fragment of *Scaf_7*, which is 400-540-bp long, only one

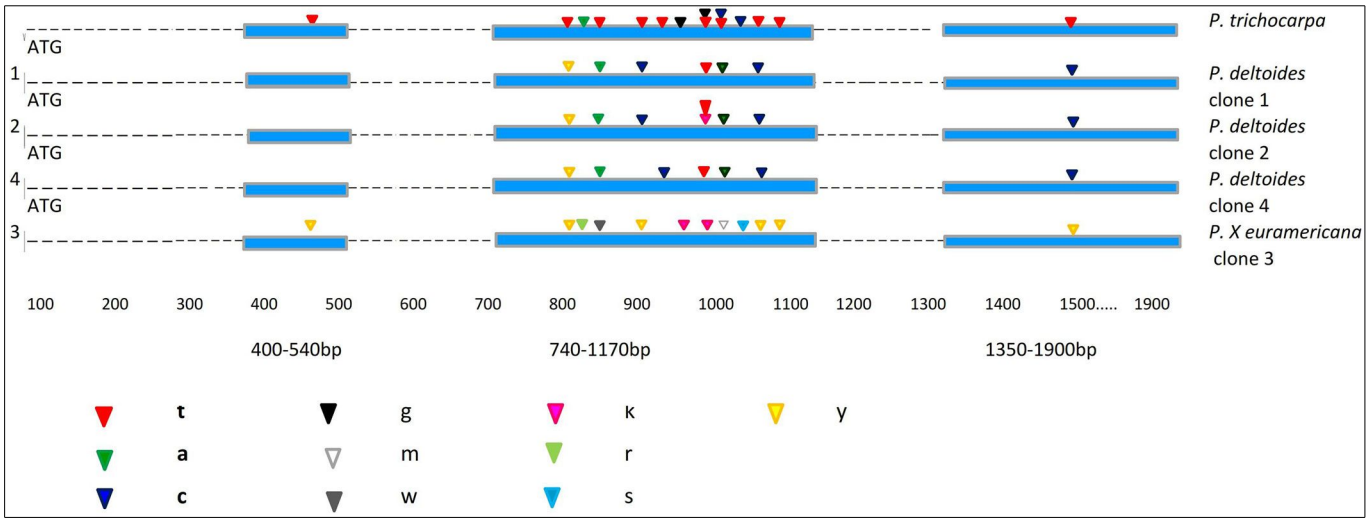

Fig. 1 - Localization of fixed nucleotide differences for the *Scaf_7* locus between the four analysed poplar clones and *P. trichocarpa* as a reference sequence. Exons and introns are indicated by boxes and lines, respectively. The IUPAC code specification for DNA sequences is: (Y): C or T; (R): A or G; (W): A or T; (K): G or T; (S): G or C; (M): A or C.

heterozygous SNP was detected at position 475 in the M1 hybrid clone. This position may be used in the differentiation of *Populus* clones and in the characterization of the M1 hybrid. The second expressed sequence analysed had the majority of SNPs and heterozygous positions (Fig. 1). In this fragment (740-1170 bp) all three *P. deltoides* clones revealed a SNP at bp 861, while only the hybrid *P.* × *euramericana* was heterozygous. This fragment is highly polymorphic, with mostly heterozygous positions found in the hybrid clone. The third fragment (1350-1900 bp) has only one position (at bp 1533) shared by all the clones, as well as the hybrid. All three species have SNPs in that position, but only the hybrid is heterozygous. This finding, *i.e.*, hybrids having more heterozygous SNPs than single species, is not surprising and is supported by several other studies (Fladung & Buschbom 2009, Chen & Polle 2010).

In the *Scaf_5* locus, more than half of the 45 polymorphic positions were heterozygous (55.5%). Of these, 10 (22.2%) belonged to the hybrid M1 clone, assuming that less than half are truly heterozygous of all the polymorphic positions. Most mutations were found to be synonymous or silent, thereby the amino acid sequence of the relative proteins does not change. In the first fragment of *Scaf_5* (150-390 bp) only one position (at bp 184) had a SNP showing a Y→T transition, which was found in the M1 hybrid clone, while a heterozygous Y→C substitution at bp 287 was shared by B229 and PE19/66 clones. Interestingly, the second expressed sequence analysed had the majority of heterozygous positions (at bp 600, 796, 797 and 814). The other two *P. deltoides* clones (B229 and PE19/66) did not show any polymorphisms.

The highest number of mutations per site was detected in the third fragment (1000-1530 bp) analysed. All four clones shared mutations at bp 1003, 1009, 1054 and 1286. In the first and second sequences of this locus, mainly heterozygous positions were found (Fig. 2). Therefore, the four poplar clones could be differentiated by "one-nucleotide SNPs".

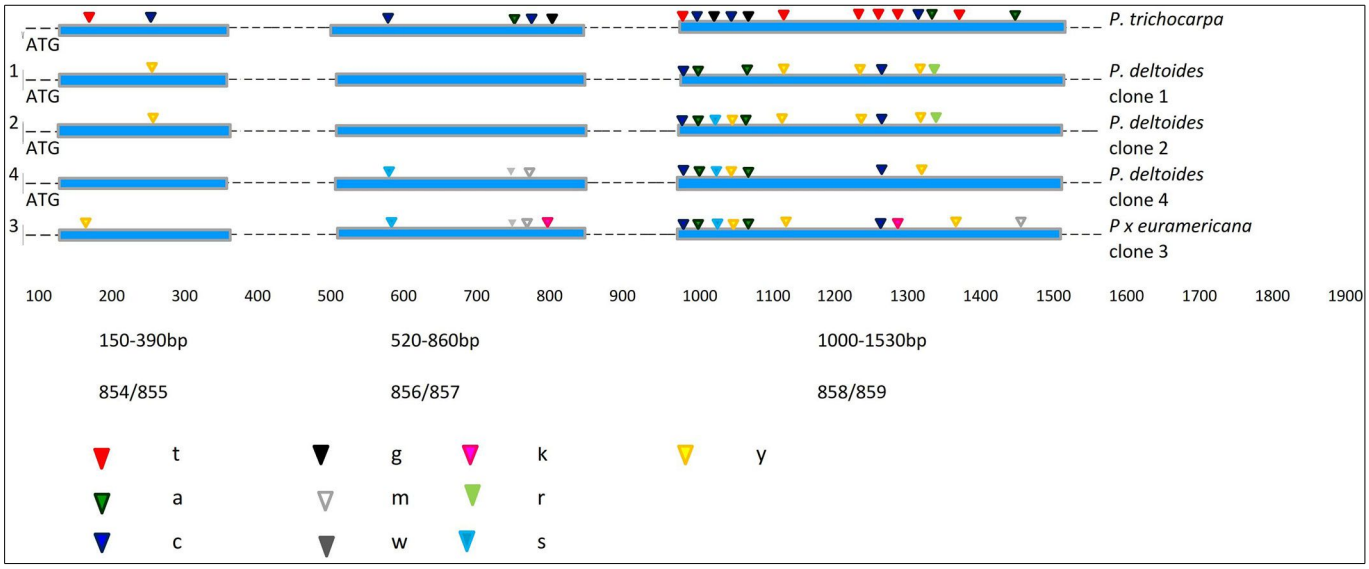

Fig. 2 - Localization of fixed nucleotide differences for the *Scaf_5* locus between the four analysed poplar clones and *P. trichocarpa* as a reference sequence. Exons and introns are indicated by boxes and lines respectively. The IUPAC code specification for DNA sequences is: (Y): C or T; (R): A or G; (W): A or T; (K): G or T; (S): G or C; (M): A or C.

Discussion

The present study provides the first insights into nucleotide polymorphisms of the *GRAS/SCL TF* gene among *Populus* clones and species. The four poplar clones selected are of high economic importance to Serbia, as they are frequently used in plantations. All four clones are characterized by varying degrees of salt tolerance, with clone M1 being the most tolerant. Both *Scaf_5* and *Scaf_7* revealed high polymorphisms in the consensus sequences of different conserved parts of the exons. This high nucleotide diversity in homologous sequences of the exons of the *GRAS/SCL TF* could have consequences for abiotic stress-resistance of different poplar clones. Indeed, Galovic & Szabados (unpublished data) used quantitative PCR to measure gene expression, finding a significant induction of *Scaf_7* during salt stress, while *Scaf_5* was expressed at a lower level during salt stress.

Our results indicate a high genetic similarity of the investigated clones to *P. trichocarpa*. As the latter species belongs to the *Tacamahaca* section, a close genetic relationship between the two sections (*Aigeiros* and *Tacamahaca*) is likely, as supported by similar findings in the literature. For example, Cervera et al. (2005) suggested close relationships between the *Leucoides, Tacamahaca* and *Aigeiros* sections according to phylogenetic analyses. Our results are also consistent with the findings of Fladung & Buschbom (2009) and Dvornyk et al. (2002), and further support a genetic similarity, to some extent, between the different investigated clones and *P. trichocarpa*. Moreover, these findings confirm the results reported by Cervera et al. (2005), who found a close relationship between *P. deltoides* (section *Aigeiros*) and species of the *Tacamahaca* section. Furthermore, Galovic et al. (2004), Galovic & Orlovic (2007) and Orlovic et al. (2009) also supported the findings of genetic similarities between two different species of the *Aigeiros* and *Tacamahaca* sections using AFLPs and SSR nuclear markers.

As a next step, the selected candidate *GRAS/SCL TF* genes and the identified SNP markers should be tested as diagnostic tools in a larger collection of salt-sensitive and salt-tolerant poplar lines, and their ability to increase salt tolerance should be verified using genetic engineering tools.

Conclusions

The *GRAS/SCL* gene is a promising candidate for improving salt stress tolerance in the poplar clones under investigation. Successful amplification of the *GRAS/SCL TF* gene and the resulting different SNP patterns detected allowed to detect differences in the genetic background of clones belonging to *P. deltoides.* The different fingerprinting patterns observed indicate that each clone could have a different potential for stress tolerance.

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