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Genome-wide identification and characterization of the MADS-box gene family in *Salix suchowensis*

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MADS-box genes encode transcription factors that participate in various plant growth and development processes, particularly floral organogenesis. To date, MADS-box genes have been reported in many species, the completion of the sequence of the willow genome provides us with the opportunity to conduct a comprehensive analysis of the willow MADS-box gene family. Here, we identified 60 willow MADS-box genes using bioinformatics-based methods and classified them into 22 M-type (11 Mα, 7 Mβ and 4 Mγ) and 38 MIKC-type (32 MIKCc and 6 MIKC*) genes based on a phylogenetic analysis. Fifty-six of the 60 SsMADS genes were randomly distributed on 19 putative willow chromosomes. By combining gene structure analysis with evolutionary analysis, we found that the MIKC-type genes were more conserved and played a more important role in willow growth. Further study showed that the MIKC* type was a transition between the M-type and MIKC-type. Additionally, the number of MADS-box genes in gymnosperms was notably lower than that in angiosperms. Finally, the expression profiles of these willow MADS-box genes were analysed in five different tissues (root, stem, leave, bud and bark). This study is the first genome-wide analysis of the willow MADS-box gene family, and the results establish a basis for further functional studies of willow MADS-box genes and serve as a reference for related studies of other woody plants.

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Abstract: MADS-box genes encode transcription factors that participate in various plant growth and development processes, particularly floral organogenesis. To date, MADS-box genes have been reported in many species, the completion of the sequence of the willow genome provides us with the opportunity to conduct a comprehensive analysis of the willow MADS-box gene family. Here, we identified 60 willow MADS-box genes using bioinformatics-based methods and classified them into 22 M-type (11 Mα, 7 Mβ and 4 Mγ) and 38 MIKC-type (32 MIKCc and 6 MIKC*) genes based on a phylogenetic analysis. Fifty-six of the 60 SsMADS genes were randomly distributed on 19 putative willow chromosomes. By combining gene structure analysis with evolutionary analysis, we found that the MIKC-type genes were more conserved and played a more important role in willow growth. Further study showed that the MIKC* type was a transition between the M-type and MIKC-type. Additionally, the number of MADS-box genes in gymnosperms was notably lower than that in angiosperms. Finally, the expression profiles of these willow MADS-box genes were analysed in five different tissues (root, stem, leave, bud and bark). This study is the first genome-wide analysis of the willow MADS-box gene family, and the results establish a basis for further functional studies of willow MADS-box genes and serve as a reference for related studies of other woody plants.

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1. Introduction

MADS-box genes, which are an important class of transcription factors in eukaryotes, are ubiquitous in animals, plants and yeast and play significant roles in the growth and development of these organisms(Alvarez-Buylla et al. 2000; Becker & Theissen 2003). In specific, these genes play an important role in myocardial development in animals, but almost all of these genes participate in all stages of growth and development in plants, particularly the development of floral organs(Zhang et al. 2017). The name MADS-box is derived from the four first letters of MCM1 from *Saccharomyces cerevisiae*, AGAMOUS from *Arabidopsis*, DEFICIENS from snapdragon and SRF4 from humans, and the proteins encoded by these genes contain a highly conserved region called the MADS-box that is approximately 60 amino acid residues in length(Messenguy & Dubois 2003).

Evolutionarily, MADS-box genes in animals, plants and fungi are divided into two major categories (type I and type II). Type I MADS-box genes are further divided into Mα, Mβ and Mγ. Type II genes, which also known as the MIKC type due to their common structure of four domains, can be further divided into two subtypes (MIKCc and MIKC*) based on different structural features(Henschel et al. 2002; Kwantes et al. 2012; Parenicova et al. 2003). Additionally, another method exists for MADS-box gene classification. For example, when the *Arabidopsis* gene family was classified, a Bayesian method was used to divide the genes into five subclasses (Mα, Mβ, Mγ, Mδ and MIKC). Structurally, almost all MADS-box genes contain a conserved MADS domain consisting of 60 amino acid residues at the N- terminus, and this domain is responsible for binding the CArG-box ($CC(A/T)_6GG$) in the regulatory region of target genes(Messenguy & Dubois 2003).

The main difference between plant type I and type II MADS-box genes is whether they contain a K domain. Type I MADS-box genes contain only one highly conserved MADS domain with no or few introns, and their abundance is lower at the transcriptional level. Type II MADS-box genes have a multi-intron structure with the exception of the highly conserved MADS domain. In order from the N- to the C-terminus, this gene type also contains the intervening (I) domain, keratin (K) domain, and C-terminal (C) region(De Bodt et al. 2003; Smaczniak et al. 2012). The I domain is a non-conserved region composed of 31-35 amino acid residues that assists with the binding to form dimers and complexes with DNA. The K domain is the second conserved region following the MADS domain and is a coiled coil with a length of approximately 70 amino acid residues. This domain is a structural unit responsible for dimerization and is also considered a characteristic sequence of MADS-box transcription factors in plants (K domains only exist in plants)(Wu et al. 2006). The C-terminal region is the most variable region and has been validated to play an important role in the formation and transcriptional activation of protein complexes.

In view of the important role of the MADS-box gene family in the plant lifecycle, researchers have identified this gene family in a variety of plants, including *Arabidopsis thaliana*, *Oryza*

sativa, *Brachypodium distachyon*, *Malus domestica*, *Ziziphus jujube*, and *Populus trichocarpa*(Arora et al. 2007; Bi et al. 2016; Kaufmann et al. 2005; Leseberg et al. 2006; Ng & Yanofsky 2001; Parenicova et al. 2003; Tian et al. 2015; Wei et al. 2014; Zhang et al. 2017). Willow is a general term for the type of woody plants belonging to the genus *Salix*, which include deciduous shrubs and arbors with a long cultivation history in China. Because of their strong adaptability to the environment and short generation period, willows have been widely recognized as an important renewable source of bioenergy that can be used in cogeneration to meet today's rapidly increasing demand for renewable resources. In addition, willows have good economic value; for example, they can be used to make boxes and process antirheumatic Chinese medicinal herbs and are cultivated as ornamental trees(Bi et al. 2016; Kuzovkina & Quigley 2005). However, the MADS-box gene family in willows has not been identified. After the draft of the *Salix suchowensis* genome sequence was completed in 2014, approximately 96% of the genetic loci were effectively annotated, and transcriptome data became easily available(Dai et al. 2014). Therefore, we have the opportunity to identify the MADS-box gene family from the willow whole-genome protein data.

Based on the latest published *Salix suchowensis* genome database, we identified members of the MADS-box gene family and analysed their chromosomal locations, exon-intron structures, evolution and gene expression profiles. These results establish a basis for further functional studies of willow MADS-box genes and serve as a reference for related studies of other woody plants.

2. Materials and Methods

2.1 Datasets and sequence retrieval

All the latest version files related to the *Salix suchowensis* genome sequence that were used for the identification of MADS-box genes were downloaded from the website of the Bioinformatics Laboratory of the Information College of Nanjing Forestry University (http://bio.njfu.edu.cn/static/ss_wrky/). *Arabidopsis* genomic data and 89 MADS-box sequences were downloaded from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/index.jsp) with the accession numbers reported by Parenicová et al., and the MADS-box protein data for rice were obtained from the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu/index.shtml)(Kawahara et al. 2013; Parenicova et al. 2003).

2.2 Identification and distribution of MADS-box genes in willows

The method used to identify proteins corresponding to the willow MADS-box genes was similar to that used for other species(Duan et al. 2015; Tian et al. 2015; Wei et al. 2014). Fasta and Stockholm format files for the MADS-box domains were retrieved from the Pfam database (release 31.0, http://pfam.xfam.org/) with the accession number 'PF00319'(Finn et al. 2016). To obtain potential proteins, an alignment of MADS-box seed sequences in the Stockholm format was

generated by a tool in the HMMER programs (hmmbuild) to build an HMM model, and then the model was used to search all willow proteins using another tool (hmmsearch) with the default parameters(Eddy 1998). Blastp (E-value = 1^{-3}) was used to align the Fasta profile downloaded from the PFAM website with all willow protein sequences (Willow.gene.pep)(Camacho et al. 2009). The potential willow MADS-box genes were obtained by taking the intersection of the above two results. To validate the confidence of these genes, we used the SMART programme (http://smart.embl-heidelberg.de/) to confirm whether a MADS-box domain was contained in each candidate MADS-box protein(Letunic et al. 2015). Genes that did not contain an entire MADS domain were removed to identify eligible MADS-box gene family members. In addition, we used the ExPasy tool (http://au.expasy.org/tools/pi_tool.html) to calculate the lengths, molecular weights, and isoelectric points of these putative MADS-box proteins. Finally, all identified MADS-box genes were mapped onto willow chromosomes with an in-house Perl script (http://bio.njfu.edu.cn/willow_chromosome/BuildGff3_Chr.pl). The distribution of each MADS-box gene on the willow chromosomes was plotted using the MapInspect software (http://mapinspect.software.informer.com/), and these genes were renamed based on their chromosomal distributions.

2.3 Multiple alignment and phylogenetic analysis of the willow MADS-box genes

The sequence logo of the identified willow MADS-box genes was generated using the web-based application WebLogo3 (http://weblogo.threeplusone.com) with the default parameters(Crooks et al. 2004). To obtain the conserved MADS-box domains of these willow MADS-box genes, we employed the online tool SMART and the PFAM database and used ClustalX (version 2.1) to perform multi-sequence alignment of the MADS-box domains obtained from SMART(Larkin et al. 2007). The online tool BoxShade (http://www.ch.embnet.org/software/BOX_form.html) was then used to colour the resulting alignment.

In general, all SsMADS genes can be divided into two categories (M-type and MIKC-type) through the PlantTFDB website (http://planttfdb.cbi.pku.edu.cn/). However, to obtain a better subgroup classification of these genes, a multiple sequence alignment including willow (SsMADS) and *Arabidopsis* (AtMADS) MADS-box proteins was performed using Muscle, and a NJ tree was built with MEGA 7.0 based on this alignment(Edgar 2004; Jin et al. 2014; Kumar et al. 2016). A NJ tree was then established for all *Arabidopsis* MADS-box proteins to check the reliability of this method(Duan et al. 2015). A phylogenetic tree was constructed using a similar method with the identified SsMADS domains and 66 rice MADS-box core domains (OsMADS). Additionally, a phylogenetic tree was built based on the identified SsMADS proteins.

Subsequently, to enable better comparison of MADS-box genes in Salicaceae, a phylogenetic tree was established for all SsMADS and *Populus trichocarpa* MADS-box genes. The method was consistent with that described above.

Finally, the orthologues of each SsMADS gene in *A. thaliana*, rice and *Populus* were

144 determined based on the phylogenetic trees of the MADS-box domains or proteins and the
145 BLASTP programme results (bi-direction, best hit, E-value = $1e^{-20}$)(Chen et al. 2007).

146 *2.4 Gene structure analysis of the willow MADS-box genes*

147 The intron-exon structures of the willow MADS-box genes were contained in our own
148 assembled protein annotation file. After annotation information for all SsMADS genes was
149 extracted using a Perl language script, an intron-exon structure diagram was obtained from the
150 online tool GSDS (Gene Structure Display server, http://gsds.cbi.pku.edu.cn/)(Hu et al. 2015).
151 Multi-sequence and Blastp alignments (E-value = 1e-20) were performed to obtain the
152 similarities between these SsMADS genes. To estimate gene duplication events in the SsMADS
153 genes, the following metrics were set: (1) the proportion of regions used for alignment of the longer
154 gene should exceed 65% and (2) the similarity of the aligned regions should exceed 65%(Bi et al.
155 2016).
156 To better reveal the structural features of the SsMADS proteins, the online tool Multiple
157 Expectation Maximization for Motif Elicitation (MEME, http://meme-suit.org/) was used to
158 predict conserved motifs in the encoded SsMADS proteins(Bailey et al. 2006). The parameters
159 were set to a repeat motif site of any number, a maximum number of motifs of 15, and a width of
160 each motif ranging from 6 to 60 residues. The web-based software 2ZIP
161 (http://2zip.molgen.mpg.de/) was used to verify whether these SsMADS proteins contained the
162 Leu zipper motif, and other important conserved motifs, including LXXLL and LXLXLX, were
163 searched manually(Bornberg-Bauer et al. 1998).

164 *2.5 Expression analysis of the willow MADS-box genes*

165 The BWA programme was used to map back the *S. suchowensis* RNA-Seq reads from five
166 tissues (roots, stems, leaves, buds and skins) onto the SsMADS gene sequences, and the number
167 of mapped reads for each SsMADS gene in RPKM (reads per kilo base per million mapped reads)
168 was calculated manually and standardized using Log_2 RPKM(Li & Durbin 2009; Wagner et al.
169 2012). A gene expression profile heat map was drawn with Bioconductor (pheatmap
170 package)(Gentleman et al. 2004).

171 **3. Results and discussion**

172 *3.1 Identification and characterization of the MADS-box gene family in Salix suchowensis*

173 Sixty-four MADS-box genes were obtained using the HMMER toolkit to search the Hidden
174 Markov Model of the MADS-box DNA-binding domain in the willow whole-genome protein
175 sequence. The accuracy of the results was verified through BLASTP and HMMER mutual
176 verification. Subsequently, the potential MADS-box genes were submitted to the SMART website
177 for further verification. Four genes were removed due to lack of a MADS domain, and the
178 remaining 60 probable MADS-box genes were selected as MADS-box superfamily members.

179 To better understand the MADS domain of *Salix suchowensis*, a sequence logo and a multiple
180 alignment with 60 SsMADS domains were generated. Amino acids 3, 23, 24, 27, 30, 31, and 34
181 were highly conserved, which confirmed conservation of the MADS domain (Figure S1).
182 As shown in Figure 1, the structures of the type I and type II SsMADS genes were quite
183 different, and the type II SsMADS genes were more conserved than the type I genes. The MIKCc
184 subgroup was the most conserved type, and several conserved motifs, including RQVT and RIEN,
185 were concentrated at the N-terminus. The similarities between types I and II mainly occurred in
186 the central region near the C-terminus. For example, differences in the N-terminal amino acids in
187 *Physcomitrella patens* were reported to determine the differences between type I and type II
188 MADS-box genes, whereas MIKCc and MIKC* are distinguished by the C-terminus(Henschel et
189 al. 2002). In general, the type II MADS-box genes of *Salix suchowensis*, particularly the MIKCc
190 subgroup, were more conserved, which indicated that the MIKCc genes might have been subjected
191 to greater selection pressure during evolution and are more important for the environmental
192 adaptability of plants.
193 Detailed characteristics, including the classification, chromosomal distribution, homologous
194 genes, and related physicochemical properties, of the SsMADS genes are listed in Table 1. As
195 shown in Table 1, these protein sequences ranged from 80 amino acids (SsMADS34) to 894 amino
196 acids (SsMADS40), with an average of 277 amino acids. Furthermore, the range of isoelectric
197 points (PIs) also showed a large fluctuation, from 4.44 (SsMADS23) to 10.33 (SsMADS34), and
198 the molecular weights (MWs) ranged from 9.20 kDa (SsMADS34) to 98.51 kDa (SsMADS40).
199 These findings reflect the high complexity of willow MADS-box genes.

200 *3.2 Chromosome distribution characteristics of the willow MADS-box genes*

201 Fifty-six of the 60 SsMADS genes were randomly distributed on 19 putative willow
202 chromosomes, and these genes were renamed SsMADS1 to SsMADS56 based on their locations
203 on the chromosomes. Only four SsMADS genes (willow_GLEAN_10001835,
204 willow_GLEAN_10001302, willow_GLEAN_10001292, and willow_GLEAN_10000968) could
205 not be mapped onto any chromosome, and these were renamed SsMADS57, SsMADS58,
206 SsMADS59, and SsMADS60, respectively.
207 As demonstrated in Figure 2, chromosomes (Chr) 1 and 2 contained the largest number of
208 SsMADS genes (six genes per chromosome), followed by Chr7, Chr8 and Chr9 (five genes per
209 chromosome). Four SsMADS genes were found on Chr3 and Chr10, and three were found on
210 Chr4, Chr6 and Chr16. Additionally, three chromosomes (Chr14, Chr15, and Chr17) contained
211 two SsMADS genes, whereas only one SsMADS gene was found on Chr5, Chr11, Chr12, Chr13,
212 Chr18 and Chr19.
213 The distribution of the MADS-box genes was not random; instead, an enrichment region
214 showed a relatively high density on some chromosomes or chromosome fragments. Previous
215 studies showed that a single chromosome region within 200 kb that contained two or more genes
216 could be defined as a gene cluster. Genes that are used in large amounts are clustered in the genome

217 to facilitate the rapid synthesis of large numbers of transcripts, which is important for predicting
218 the potential function of co-expressed or clustered genes in angiosperms.
219 According to the present study, a total of 21 SsMADS genes in willows were clustered into
220 11 clusters and distributed on nine chromosomes (Figure 2). Two gene clusters were found on
221 Chr1, including four SsMADS genes; one gene cluster each was distributed on Chr2, Chr3, Chr4,
222 Chr7, Chr8, Chr9, Chr14 and Chr17. Three SsMADS genes were distributed in the gene cluster on
223 Chr3, whereas no gene cluster was found on the other ten chromosomes. We hypothesized that
224 these clustered genes play more important roles in the growth and development of willows; as a
225 result, the clustered distribution of these genes might have given them a selective advantage during
226 evolution, and selection could have maintained the existence of the gene clusters. For example,
227 clustered genes co-expressed in yeast maintain a good co-expression relationship in
228 nematodes(Hurst et al. 2002).
229 However, the chromosomal distribution of the gene clusters was irregular. Related studies
230 have suggested that the exact position and orientation of these clustered genes are not well
231 conserved(Lee & Sonnhammer 2003).

232 *3.3 Classification of MADS-box genes in willows*

233 To better classify these SsMADS genes, a phylogenetic tree (NJ tree) was constructed using
234 88 AtMADS proteins from *A. thaliana* and the 60 SsMADS proteins identified in the present study.
235 Based on the phylogenetic tree and structural features of the MADS-box proteins, all 60 SsMADS
236 genes could be divided into two main groups (type I and type II) (Figure 3).
237 A total of 22 members were classified as type I (M-type), and these were further classified
238 into Mα, Mβ and Mγ, with 11, 7 and 4 members each, respectively. The remaining 38 members
239 were categorized as type II (MIKC-type), which included 32 MIKCc-type and 6 MIKC*-type
240 members. During the analysis, we found that SsMADS56 did not contain a K domain but was
241 divided into the MIKCc subgroup and clustered with SsMADS58. Further research found that
242 although this gene did not have a K domain, it contained an FMO-like domain that interfered with
243 the formation of the K domain, probably because it had mutated during evolution. Similar
244 phenomena have occurred in other species, such as *P. patens*(Henschel et al. 2002). Furthermore,
245 a similar classification was obtained with the NJ tree established for the 60 SsMADS domains and
246 66 rice MADS domains (Figure S2).
247 To better investigate the role of MADS-box genes in Salicaceae, we constructed a
248 phylogenetic tree using 103 poplar and 60 willow MADS domains (Figure S3). Based on the NJ
249 tree described above, we found that most of the MADS-box genes from willows and poplars were
250 clustered into sister pairs (40 SsMADS genes, accounting for 66.7% of all willow MADS-box
251 genes, such as SsMADS32-PtMADS12 and SsMADS37-PtMADS89) because they originated
252 from a common ancestor.
253 After the evolution analysis, we found that the MIKC* (Mδ) class was a transition subgroup
254 for the type I and type II willow MADS-box genes. As shown in the phylogenetic trees described

above, these genes were clustered between the type I and type II genes: most of them were classified as type I, but some were categorized as type II, which might be due to the more recent emergence of type I genes compared with type II genes. The MIKC*(Mδ) class represented a transition from type II to type I during evolution that had characteristics of the two types of SsMADS genes. This phenomenon has also been found in cucumbers, poplars and other species(Hu & Liu 2012; Leseberg et al. 2006).

Compared with those in poplar, the MIKC*(Mδ) genes in willows were almost completely clustered in the type I cluster, which suggested that the evolution rate of willows was faster than that of poplars.

In addition, we compared the number of willow MADS-box genes with those of the ancient tree species *Ginkgo biloba*. The *G. biloba* MADS-box genes were predicted using the same method used to predict the willow MADS-box genes. The results revealed that *G. biloba* contained only 26 MADS-box genes, which was quite different from the number found in the willow genome. The number of MADS-box gene family members of gymnosperms, such as the pine tree, an angiosperm variety, as well as monocotyledonous plants, such as corn and rice, and dicotyledons, such as apples and soybeans, were also analysed (Table 2). The gymnosperm genome was larger, but the number of this gene family was much smaller than that of the angiosperms. We speculate that this phenomenon occurred because the MADS-box gene family mainly acts on the growth and development of flower organs, and gymnosperms generally have no obvious flowers. In contrast, angiosperms, which are also called flowering plants, have a wide variety of flowers. Therefore, the number of MADS-box genes in gymnosperms was significantly smaller than that in angiosperms.

3.4 Orthologues of SsMADS genes in Arabidopsis, rice and poplars

The clustering of orthologous genes emphasizes the conservation and divergence of gene families that might have the same functions. Specifically, the clustering of orthologous genes suggests that they might have the same or similar functions(Ling et al. 2011). In this study, orthologous SsMADS genes in *A. thaliana*, rice and poplar were identified through a phylogenetic analysis combined with a BLAST-based method (bi-direction best hit). Finally, 35 pairs of orthologous genes from willow and *A. thaliana*, 35 pairs from willow and rice, and 57 pairs from willow and poplar were identified. The 22 type I SsMADS genes had 20 pairs of orthologous genes in poplar and five in *A. thaliana*, whereas rice contained no orthologues of the 22 type I SsMADS genes. The 38 type II SsMADS genes had 37, 30 and 35 pairs of orthologous genes in poplar, *A. thaliana*, and rice, respectively. Due to the imbalance between types I and II, we concluded that the MIKC-type appeared earlier than the M-type and was more conserved, whereas the M-type occurred later and evolved faster. In addition, 12 SsMADS genes were found to have identical domains in poplars (SsMADS9, SsMADS14, SsMADS17, SsMADS23, SsMADS24, SsMADS26, SsMADS43, SsMADS46, SsMADS50, SsMADS51, SsMADS53 and SsMADS58), and these accounted for 20% of the total number of genes. Among these 12 genes, 11 were MIKC-type, and

293 only SsMADS23 was Mα; in addition, all 11 MIKC genes were found to have orthologous genes
294 with high similarity in *Arabidopsis* and rice. For example, the similarity between SsMADS14 and
295 OsMADS7/45 was 98.33%, the similarity between SsMADS14 and AGL2/AGL9 was 100%, the
296 similarity between SsMADS43 and AGAMOUS was 98.31%, and the similarity between
297 SsMADS50 and AGL2/AGL9 was 100%.
298 We also found that the vast majority of SsMADS genes that did not have orthologous genes
299 in *Arabidopsis* also had no orthologous genes in rice. We hypothesized that these genes might have
300 formed after species differentiation, had unique genetic characteristics of Salicaceae plants, and
301 might even be specific to Salicaceae plants, although these speculations require further research.
302 Because most of the *Arabidopsis* MADS-box genes had functional annotations, the functions of
303 the willow MADS-box genes could be predicted based on the orthologous gene pairs between
304 willows and *Arabidopsis*. Functional information for the *Arabidopsis* MADS-box genes was
305 obtained from the TAIR website. For example, the main function of the AGL2 gene in *A. thaliana*
306 is to regulate the development of flowers and ovules, and because SsMADS14/32/50/53 are
307 orthologous to this gene, it can be speculated these four genes in willow have similar functions.
308 SsMADS17 and SsMADS43 are homologous to the *Arabidopsis* AGAMOUS gene, which has a
309 primary function of specifying the floral meristem and binding to the CArG-box sequence. The
310 functions of other genes can be speculated in the same manner.

311 *3.5 Exon-intron structures of the SsMADS genes*

312 The exon-intron structures of multiple gene families play crucial roles during plant
313 evolution(Bi et al. 2016). To gain insights into the structural diversity of willow MADS-box genes,
314 we analysed the exon-intron organization of the coding sequences of each willow MADS-box
315 gene. A striking bimodal distribution of introns was observed in the *Arabidopsis*, cucumber and
316 apple MADS-box family genes; the MIKCc and MIKC*(Mδ) genes contained multiple introns,
317 whereas the Mα, Mβ, and Mγ genes usually had either no or a single intron(Hu & Liu 2012;
318 Parenicova et al. 2003; Tian et al. 2015). We found a similar finding in willow. In Figure 4, the
319 SsMADS gene phylogenetic tree and the corresponding exon-intron structures are shown in the
320 left and right panels, respectively. Among the 38 MIKC-type members, 34 (89%) members
321 contained at least four introns, and the maximum of 13 introns was detected in SsMADS40.
322 Correspondingly, among the 22 M-type genes, most of the members had no intron (77%) or a
323 single intron, especially the Mγ-type SsMADS genes, and none of these four genes had any introns.
324 Regardless, we found seven introns in SsMADS6 and eight introns in SsMADS8.
325 The following interesting phenomenon was also observed: the number of introns in the six
326 MIKC*-type willow MADS-box genes was quite varied. Among these genes, SsMADS40
327 contained 13 introns, SsMAADS26 contained 10 introns, SsMADS31 contained nine introns,
328 SsMADS28 contained four introns, and SsMADS34 and SsMADS56 contained only one intron
329 each. This dramatic change in the number of introns indicated that they were acquired or lost
330 during evolution of the MIKC*-type willow MADS-box genes. The intron numbers of the MIKCc-

type SsMADS genes were relatively stable, and further analysis showed that the intron positions of the MIKCc-type SsMADS genes were also highly conserved; this phenomenon also occurred in cucumbers, probably because these genes were purified during evolution and were more stable against environmental stress(Hu & Liu 2012).

3.6 Gene duplication events and conserved motifs in willows

Gene duplication events have always been considered vital sources of biological evolution(Chothia et al. 2003). Two or more adjacent homologous genes located on a single chromosome are considered tandem duplication events (TDs), whereas homologous gene pairs between different chromosomes are defined as segmental duplication events (SDs)(Bi et al. 2016; Liu & Ekramoddoullah 2009). In this study, we identified a total of 12 homologous gene pair (including 24 SsMADS genes) duplication events. Among them, 20 genes were MIKC-type genes (18 MIKCc and two MIKC*), and the remaining four genes were classified as Mα (Table S1). This finding suggested that the functions of the MIKC type, particularly the MIKCc type, were strengthened and played more important roles in willow evolution.

Among the 12 homologous gene pairs, two appeared to have undergone TDs, and ten participated in SDs, implying that the expression of the MADS-box gene family in willows was affected by both tandem and segmental duplication events. In contrast, the effect of SD events was greater than that of TDs, which might be due to genome-wide duplication.

The conserved motifs of the 60 MADS-box proteins were predicted by the MEME programme to better analyse the sequence characteristics and structural differences among these genes. A total of 15 conservative motifs were predicted, and named from Motif 1 to Motif 15 (Figure 5, Table S2).

Among these, Motif 1 and Motif 3 were widely present in all SsMADS genes. These two motifs were MADS domains, and Motif 1 was the most typical MADS domain. Motif 2 was a highly conserved K domain motif that is essential for protein interactions between MADS-box transcription factors and was present in all MIKC-type SsMADS genes except SsMADS44 and SsMADS56. Interestingly, the K-box domain was identified in SsMADS44 using the SMART programme but was not found using MEME because the two programmes used different algorithms. Further observation revealed that the K-box domain of SsMADS44 consisted of only 53 amino acids, whereas most K-box domains in willows were 92-93 amino acids in length; this shorter length might have been due to loss of a portion of the gene during evolution, which resulted in its distinctive features. Overall, SsMADS genes of the same subgroup had similar motifs, and we speculated that they might have similar functions. A total of six basic leucine zipper (bZIP) motifs were found in five SsMADS (SsMADS9, SsMADS16, SsMADS18, SsMADS 19, and SsMADS46) using 2ZIP, and these motifs play important roles in the expression and regulation of higher plant genes. The activation domain LXXLL motif and the inhibitory domain LXLXLX motif were also found in willow MADS-box genes. In general, a large number of motifs with different structures and functions were found in the willow MADS-box gene family, indicating

369 that the MADS-box genes play a variety of important roles in the gene regulatory network of
370 willows.

371 *3.7 Expression profiles of willow MADS-box genes in different tissues*

372 To obtain more information regarding the roles of MADS-box genes in willows, RNA-Seq
373 data from the sequenced genotype were used to quantify the expression levels of MADS-box genes
374 in five tissues from *S. suchowensis*. The expression profile heat map of 60 SsMADS genes drawn
375 using R is shown in Figure 6; the red blocks indicate high expression, the blue blocks indicate low
376 expression, and the light-green blocks indicate that the gene is not expressed in this tissue. As
377 illustrated in Figure 6 and Table S3, most of the MADS-box genes were expressed at low levels
378 or not expressed in these five tissues; this pattern was similar to the expression patterns of the
379 MADS-box gene family in *Medicago truncatula*, in which seven of the genes, including
380 SsMADS3, SsMADS12, and SsMADS18, were not expressed in the five tissues(Zhang et al.
381 2014). In contrast, 26 SsMADS genes were expressed in all tissues, and eight genes, including
382 SsMADS9, SsMADS16, and SsMADS23, were highly expressed. SsMADS9 exhibited the highest
383 expression level in four tissues (root, stem, leaf and bud) and showed high expression in bark. The
384 gene belonging to the highly conserved MIKCc type, which can be considered the housekeeping
385 gene of *S. suchowensis*, participates in various growth and development processes. SsMADS37
386 exhibited the highest expression in bark but quite low expression in the other four tissues.
387 Additionally, seven of the eight genes with higher expression were of the MIKC type; six of these
388 were of the highly conserved MIKCc type, and the remaining gene was of the MIKC* type. We
389 could infer that compared with the M-type SsMADS, the MIKC-type SsMADS play more
390 important roles in willow growth and development processes. Overall, the total RPKM value of
391 the SsMADS genes was 287 in root and higher than 400 in the remaining four tissues. Therefore,
392 the expression of the SsMADS genes in root was significantly lower than that in the stem, leaves,
393 buds and bark. Thus, the MADS-box gene family plays a major role in willow morphogenesis.
394 Furthermore, we found an interesting gene, SsMADS44, which was highly expressed in the stem
395 but expressed at extremely low levels or not expressed (root) in the other four tissues. The
396 expression profiles of the MADS-box genes obtained in our study will contribute to further studies
397 of the regulation of MADS-box genes in plant growth.
398

399 **4. Conclusions**

400 Based on the latest *S. suchowensis* genome sequence and RNA-Seq data, we identified 60
401 SsMADS genes using bioinformatics methods and classified them as M-type (Mα, Mβ, and Mγ)
402 and MIKC-type (MIKC*(Mδ) and MIKCc) according to their evolutionary relationships and
403 protein structure characteristics. We found that the gene structures of these two types were quite
404 different, which was consistent with the results of previous research in other species. Further
405 bioinformatics analyses performed for the obtained gene family members showed that the MIKC*

406 (Mδ) subclass was a transitional class between the M and MIKC types. A comparison of the
407 numbers of MADS-box genes in gymnosperms and angiosperms showed that the numbers of genes
408 in gymnosperms was significantly lower than that in angiosperms, further illustrating that these
409 genes are important for the development of floral organs. In addition, after analysing the gene
410 structures, gene duplication events and motifs of *S. suchowensis*, we found that the MIKC type
411 was more conserved than the M type and plays a more important role in the growth and
412 development of *S. suchowensis*. The above results were confirmed by expression analysis of the
413 MADS-box genes in different *S. suchowensis* tissues. In summary, the results of this study
414 establish a foundation for a better comprehensive identification of MADS-box genes in *S.*
415 *suchowensis* and a better understanding of the structure-function relationship between SsMADS
416 genes. Compared with the related genera of poplar, which is the model species of woody plants,
417 willow has a shorter generation period and a higher evolutionary rate and is thus easier to study
418 (Dai et al. 2014). Our study of the willow MADS-box gene family might also provide a useful
419 genetic database for molecular analyses of woody plants.
420

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Figure 1

Comparison of the MADS-box domains from the 60 willow MADS-box genes.

The multi-alignment was performed using the ClustalX programme (version 2.1) and coloured using the online tool BoxShade (http://www.ch.embnet.org/software/BOX_form.html). Black indicates a highly conserved region.

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Mα

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SsMADS19   1 -MGRRKIEIEMVKDSNSRQVTFSKRRTGVFKKANELATLCAVQIAIIVFSPGGK--PFSFGHP
SsMADS30   1 -MGRRKIKIEKISKKNHLQVTFSKRRAGLFKKASELSTICGVDIAVIVFSPAQK--AFSFGHP
SsMADS4    1 -KGRQKIEIKRVEKESNRYVTFSKRKNGLFKKATELSTLCGAETAVIIFSEHRK--LFSCGQP
SsMADS5    1 -KGRQKIEIKRVERESNRYVTFSKRKNGLFKKATELSTLCGAETAVIIFSEHRK--LFSCGQP
SsMADS18   1 -KGRQKIAIKRIENEDDRLVTFSKRRSGIYKKASELVTLCGAEVAVLVFSPAGK--PFSFGHP
SsMADS35   1 -RGRQKVEMKRIEKEDDRLVTFSKRRAGIYKKLSELVATGAEIACLVFSPGGK--PFSYGHP
SsMADS27   1 -RGRQRLEMVKIPNDSNLMVTFSKRRSGLFKKASELSTLCGAEVTLIVFSPGKK--VFSFGHP
SsMADS38   1 -RGRQKVEMVKMSKESNLQVTFSKRRSGLFKKASELSTLCGAEIAIIVFSPGNK--VFSFGHP
SsMADS39   1 -QGRQKIEIRQLQEKSSLQVTFSKRRGGIVKKASELSLLCGAQVAILAFSPGKK--VFAFGHP
SsMADS23   1 MGRTRKIPMAKRETAEQRSVTFTKRRQGLFNKAADLCRICDAQIAIMVSSTGSKEKVYTFGHS
SsMADS55   1 ISSMADS-MARRETAKQRSVTLTKRRQGLFNKAAELCRICDARIAIMVSSTGSKEKVYAFGHS
```

Mβ

```
SsMADS29   1 ---ENNKTTK---------SYEDRRNTLKKKARELAILCDVPVCLIGVD-------PDGTPETWPE
SsMADS42   1 -SMADSMKNK---------SYEERRQTLKKKASELATLCDVPVCLVGVN-------PDGTTETWPE
SsMADS48   1 -SMKKINQGDKITR---AMSESKRQPTLKKKAEELKTLCGVTICMVGFG-------PDGTVETYPE
SsMADS52   1 ASMPNYKRKKFLTRDQAGMSESKRTKTLKKKAHEIQTLCDVKVCMVGFG-------PDSPVQTWPE
SsMADS41   1 -----KGQEK---------SYRKRQAIEKKATEIAILCDVPVCVIVKDN------TDGRVSTVGQ
SsMADS6    1 MGRGKLTMELICNERSRMITYHKRRKGLTKKAREFQILCGVDACVIILGPKQNN--HPVDVETWPT
SsMADS12   1 MGQKRIKMELIRKEKSRMLTFRKRRAGLLKKASEFSILCGVDACVIIFSPKLKDDRQSVAPETWPP
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Mγ

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SsMADS3    1 MARKKVKLMNIVNTAARKASLKKRR--DGLLKKVSELTILCGIEAFVIIYCPDDPEPAIK-----PS
SsMADS22   1 MTRKKVKLTWIVNDSARRASLKKRR--VGLLKKVSELTILCGVEAFVIIYSPDDPEPTVV-----PS
SsMADS59   1 MTRKKVKLAMITNDSARKATEKKRK--KGLMKKVSELSTLCGIEACAIICSPYDAQPEVV-----PS
SsMADS60   1 SGEHASQRSEVQNCEVKNDNIQRQQWIVDFLNPQ-EPSGFGPPEMLLPFVDNNQNP-LVSNNFFPS
```

Mδ /MIKC*

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SsMADS28   1 MGRNKIPLKKIDNPCRRKITYSKRRDGIIKKATELSVLCETEVGVLMYSPHGRLTTFSSN
SsMADS34   1 MGRLKIQLRKIENKTSRHVTFAKRRGGLVKKAYELSTLCDVEIAVIIFSPAGKLILFEGK
SsMADS26   1 MGRVKIQLKRIENNTNRQVTFSKRRNGLIKKAYELAILCDIDIALIMFSPSGRLSHFSGK
SsMADS31   1 MGRVKLKIKKLENSNGRQATYAKRKHGIMKKANELSILCDIDIILLMFSPTGKPSICKGA
SsMADS40   1 MGRVKLKIKKLENTNGRQATYAKRKHGIMKKANELSILCDIDILLLMFSPTGKPSICKGA
SsMADS57   1 MGRRKLKIQRLECVKARQAKYSKRKIGLLKKAKELAILCDIDLALVMFSPTKKPSLYVGQ
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MIKCc

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SsMADS14   1 MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSS
SsMADS50   1 MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCSS
SsMADS53   1 MGRGRVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLYEFCST
SsMADS32   1 MGRGKVELKRIENKINRQVTFAKRRNGLLKKAYELSVLCDAEVALIIFSNRGKLFEFCSS
SsMADS46   1 MGRGRVELKRIENKINRQVTFSKRRNGLLKKAYELSVLCDAEVALIIFSSRGKLYEFGSA
SsMADS2    1 MGRGKVVLERIENKISRQVTFSKRRNGLLKKAYELSLLCDAEVALIIFSSHGKLFEFGEV
SsMADS33   1 MGRGRVQLKRIENKINRQVTFSKRRAGLLKKAHEISVLCDAEVALIVFSHKGKLFEYSTN
SsMADS54   1 MGRGRVQLKRIENKISRQVTFSKRRTGLQKKAHEISVLCDADVALIVFSTKGKLFEYSID
SsMADS17   1 LGRGKVEIKRIENTTNRQVTFCKRRSGLLKKAYELSVLCDAEVALIVFSSRGRLYEYSND
SsMADS43   1 LGRGKVEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEYSNN
SsMADS15   1 MGRKKVELKRIENNSSRLVTFSKRRHGLFKKARELSVLCDVQIALLIFSSRGKLYEFSSV
SsMADS16   1 MGRKKVELKRIENNSSRLVTFSKRRQGVFKKARELSVLCDVQIALLIFSSRGKLYEFSSV
SsMADS11   1 -------MRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVAVIVFSPRGKLYEFGSS
SsMADS47   1 MVRGKTQMRRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIVFSPRGKLYEFASS
SsMADS13   1 MARGKTQMKRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIVFSTRGKLYEFSSS
SsMADS51   1 MVRGKTQMKRIENATSRQVTFSKRRNGLLKKAFELSVLCDAEVALIVFSTRGKLYEFSSS
SsMADS49   1 MVRGKIQMRRIENASSRQVTFSKRRNGLLKKARELSVLCDAEVAVIIFSQNGKLYEFAST
SsMADS1    1 MVRGKVQLKRIENATSRQVTFSKRKNGLLKKAYELSILCDAEVAVIIFSQKGTLFASADQ
SsMADS56   1 MARGKVQLKRIENQVHRQVTFCKRRAGLLKKAKELSVLCDAEIGVFIFSAHGKLYELATK
SsMADS58   1 MARGKVQMKRIENSVHRQVTFCKRRSGLLKKAKELSVLCDAEIGVFIFSAHGKLYELATK
SsMADS10   1 MGRGKIVIRRIDNPTSRQVTFSKRRNGLMKKAKELAILCDAEVGVVIFSSTGKLYDFSST
SsMADS37   1 MGRGKIVIRRIDNSTSRQVTFSKRRSGLLKKAKELSILCDAEIGVIIFSSTGKLYDYAST
SsMADS44   1 MGRGKVVLQRIENPTRRQVTFSKRRNGLLKKAFELSILCDAEVSLVIFSPTGKCYQFASH
SsMADS45   1 MGRGKVVLQRIENPTRRQVTFSKRRNGLLKKAFELSILCDAEVSLVIFSPTGKCYQFASH
SsMADS9    1 MAREKIKLKKIDNVTARQVTFSKRRRGLFKKAEELSVLCDAEVAVIIFSATGKLFEYSSS
SsMADS36   1 MGRGKIEIKKIENANSRQVTFSKRRAGLLKKAQELAILCDAEVAVIVFSNTGKLFEFSSS
SsMADS21   1 MGRGKIEIKRIENLNSRQVTFSKRRNGLLKKARELSVLCDAEVAVIIFSSTGKLYEFSST
SsMADS20   1 MGRGKIAIRRIENQTTRQVTFSKRRAGLLKKTHELSVLCDAQIGLIVFSSTGKLCQYCTE
SsMADS25   1 MGRGKIAIRRIENQTTRQVTFSKRRGGLLKKTRELSVLCDAQIGLIIFSSTGKLCEYCTE
SsMADS7    1 MARGKIQLKKIENSTNRQVTYSKRRNGLFKKAHELTVLCDAEVSLIMVSCTDKVHDYISP
SsMADS24   1 MGRGKIEIKKIENPTNRQVTYSKRRNGIFKKAQELTVLCDAKVSLIMFSNTNKLNEYISP
SsMADS8    1 MGRGKIEIKKIENSGNRQVTYSKRRSGLLKKAKELTVLCDAQVSLLMASSTRKLNEYCSP
```

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Figure 2

Chromosomal localization of the 60 willow MADS-box genes.

The number of each chromosome is given above the lines. The left side of each chromosome is related to the approximate physical location of each MADS-box gene. The four unmapped genes are shown on ChrN. Purple indicates Mα, green indicates Mβ, brown indicates Mγ, yellow indicates MIKC*, and blue indicates MIKCc.

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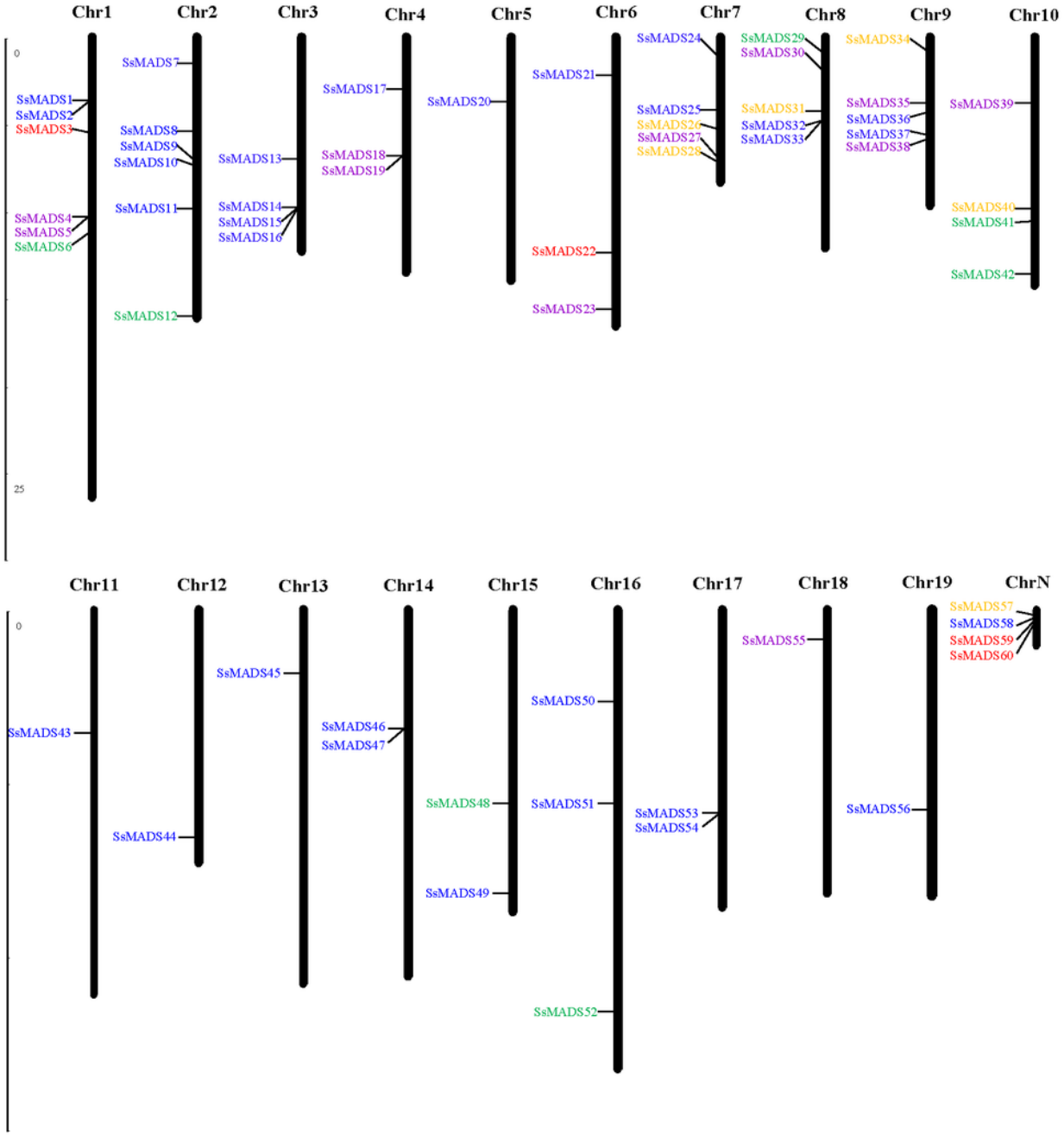

Figure 3

Phylogenetic tree of *S. suchowensis* and *A. thaliana* MADS-box proteins.

A total of 60 MADS-box proteins from *S. suchowensis* and 88 from *A. thaliana* were used to construct a NJ tree using MEGA 7. Different shapes and colours represent different species and gene categories.

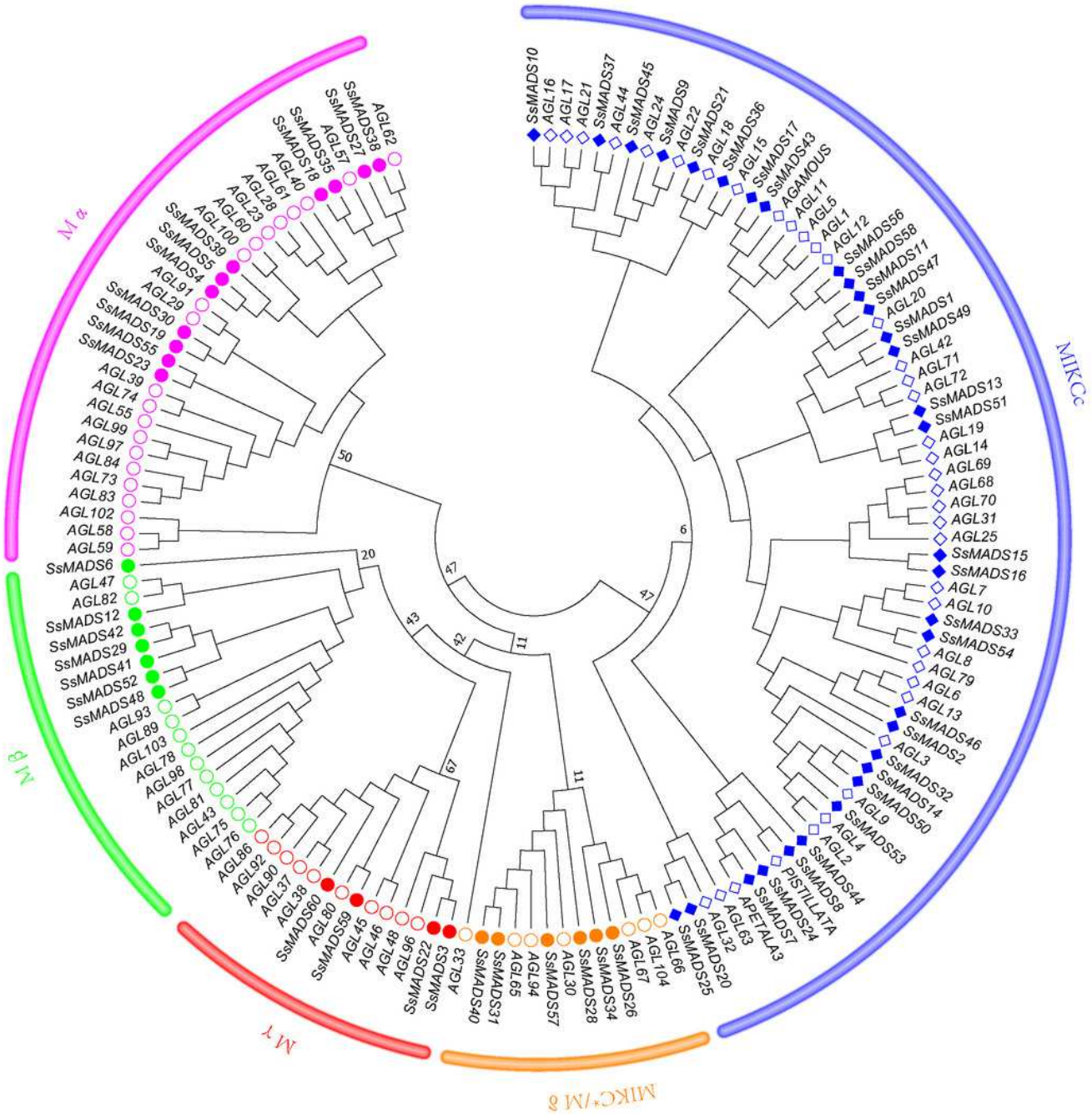

Figure 4

Phylogenetic relationships and gene structures of the willow MADS-box genes.

An unrooted NJ tree was constructed based on the full-length willow MADS-box protein sequences. The exon-intron structures of the willow MADS-box genes were displayed using the online tool GSDS.

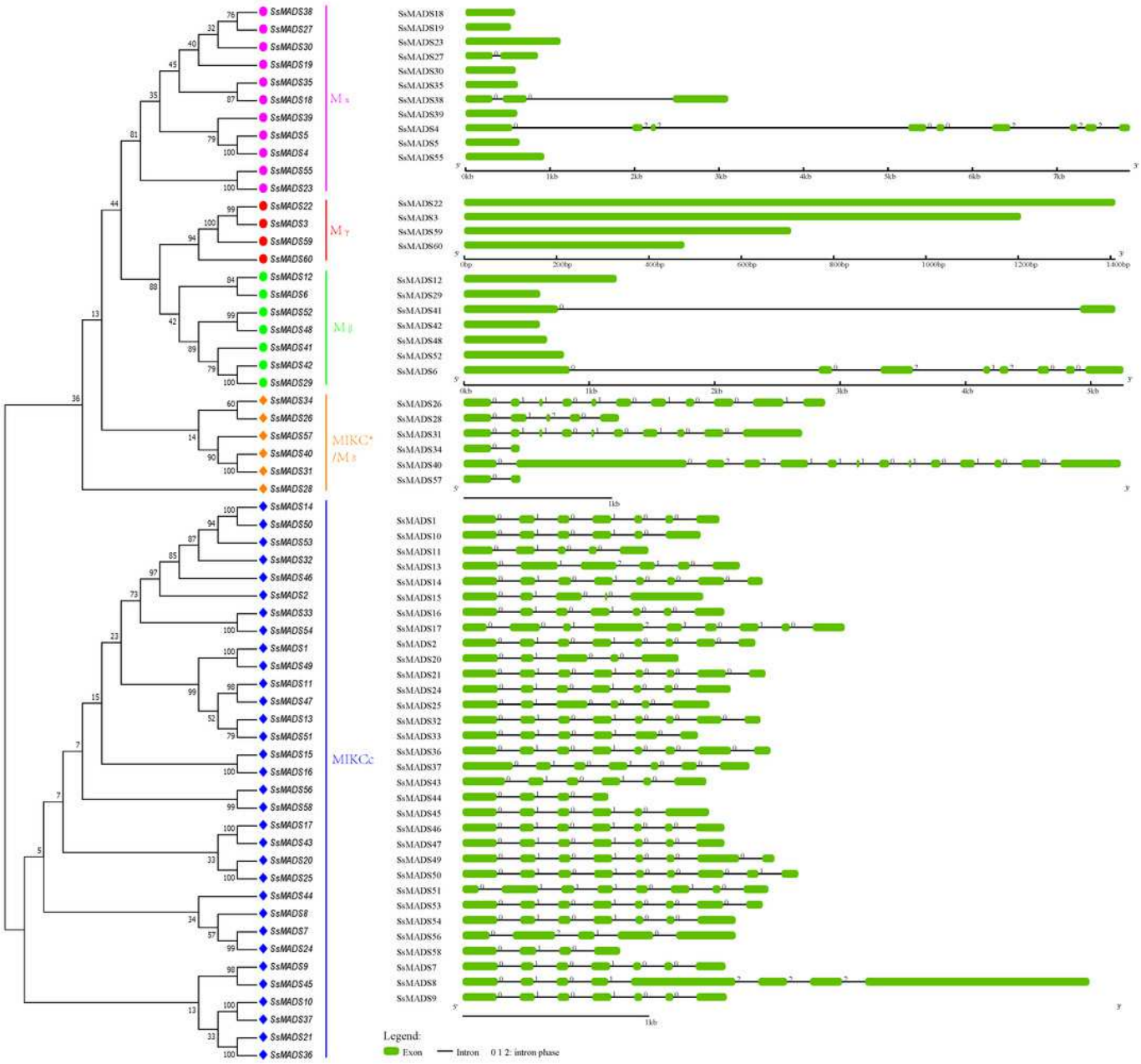

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Figure 5

Conversed motif distributions of the willow MADS-box proteins.

A total of 15 conserved motifs of the 60 willow MADS-box proteins were identified using MEME. Motifs 1-15 are indicated by different colours.

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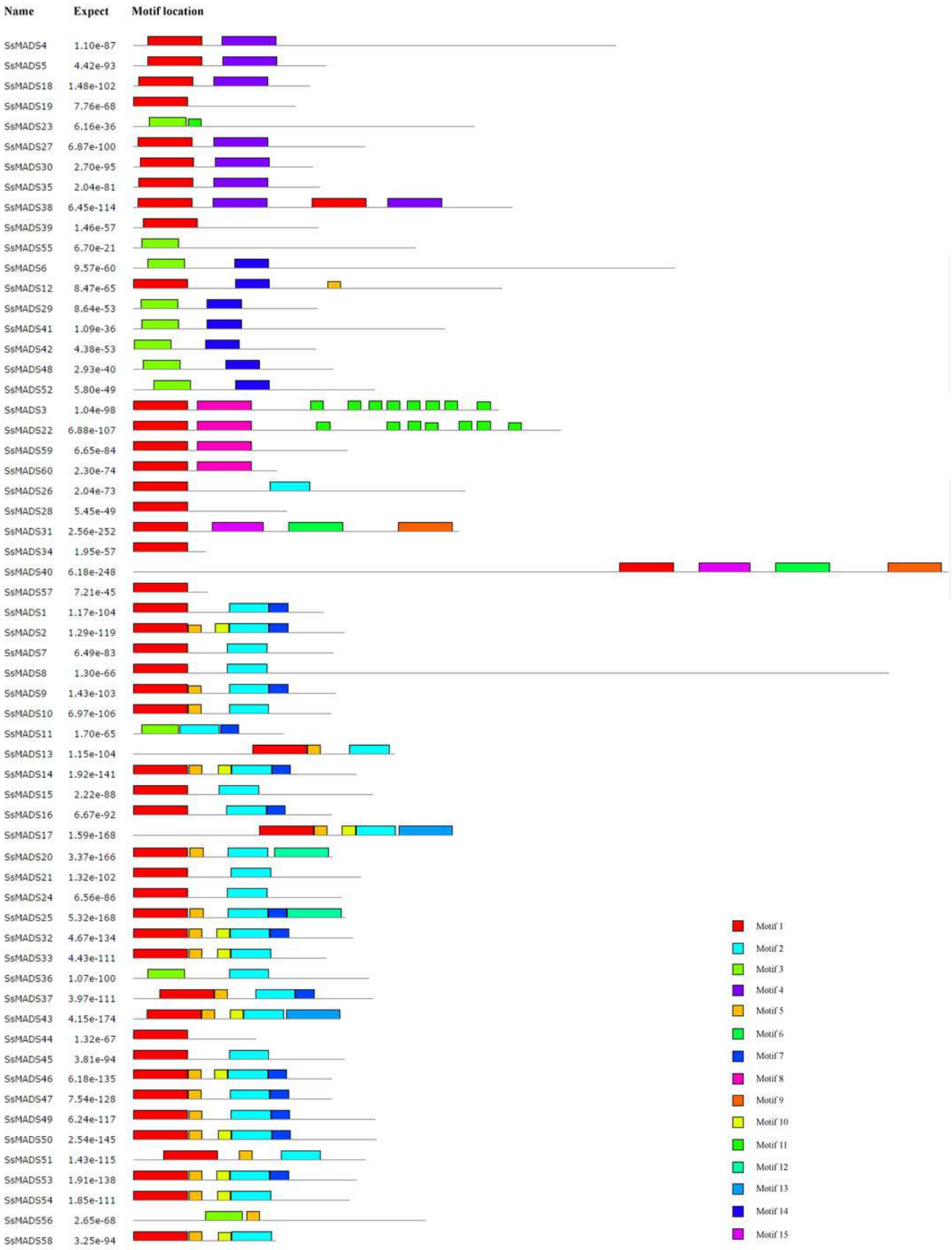
Name
Expect
Motif location
SsMADS4 1.10e-87
SsMADS5 4.42e-93
SsMADS18 1.48e-102
SsMADS19 7.76e-68
SsMADS23 6.16e-36
SsMADS27 6.87e-100
SsMADS30 2.70e-95
SsMADS35 2.04e-81
SsMADS38 6.45e-114
SsMADS39 1.46e-57
SsMADS55 6.70e-21
SsMADS6 9.57e-60
SsMADS12 8.47e-65
SsMADS29 8.64e-53
SsMADS41 1.09e-36
SsMADS42 4.38e-53
SsMADS48 2.93e-40
SsMADS52 5.80e-49
SsMADS3 1.04e-98
SsMADS22 6.88e-107
SsMADS59 6.65e-84
SsMADS60 2.30e-74
SsMADS26 2.04e-73
SsMADS28 5.45e-49
SsMADS31 2.56e-252
SsMADS34 1.95e-57
SsMADS40 6.18e-248
SsMADS57 7.21e-45
SsMADS1 1.17e-104
SsMADS2 1.29e-119
SsMADS7 6.49e-83
SsMADS8 1.30e-66
SsMADS9 1.43e-103
SsMADS10 6.97e-106
SsMADS11 1.70e-65
SsMADS13 1.15e-104
SsMADS14 1.92e-141
SsMADS15 2.22e-88
SsMADS16 6.67e-92
SsMADS17 1.59e-168
SsMADS20 3.37e-166
SsMADS21 1.32e-102
SsMADS24 6.56e-86
SsMADS25 5.32e-168
SsMADS32 4.67e-134
SsMADS33 4.43e-111
SsMADS36 1.07e-100
SsMADS37 3.97e-111
SsMADS43 4.15e-174
SsMADS44 1.32e-67
SsMADS45 3.81e-94
SsMADS46 6.18e-135
SsMADS47 7.54e-128
SsMADS49 6.24e-117
SsMADS50 2.54e-145
SsMADS51 1.43e-115
SsMADS53 1.91e-138
SsMADS54 1.85e-111
SsMADS56 2.65e-68
SsMADS58 3.25e-94
Motif 1
Motif 2
Motif 3
Motif 4
Motif 5
Motif 6
Motif 7
Motif 8
Motif 9
Motif 10
Motif 11
Motif 12
Motif 13
Motif 14
Motif 15

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Figure 6

Expression analysis of the 60 willow MADS-box genes in five tissues (bark, leaf, bud, root and stem).

The colour scale represents RPKM normalized log2-transformed counts. The red blocks indicate high expression, the blue blocks indicate low expression, and the light green blocks indicate no expression in this tissue.

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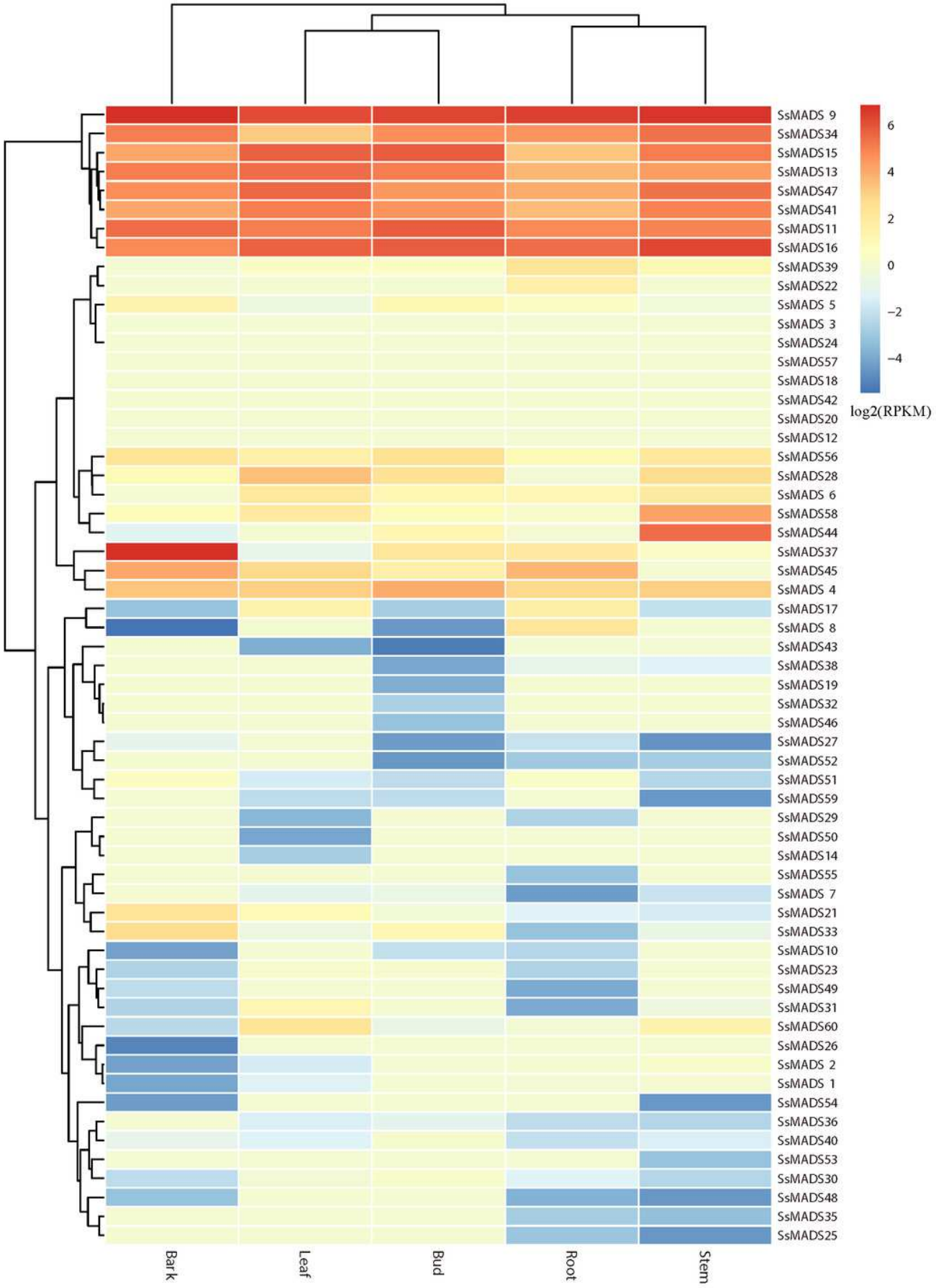

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Table 1 (on next page)

Detailed information for the MADS-box gene family in willow.

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1 **Table 1.** Detailed information for the MADS-box gene family in willow.

				Orthologue			Physicochemical characteristics			
Gene	Sequence ID	Class	Chr	PtMADS	AtMADS	OsMADS	Length (aa)	MW (kDa)	PI	Introns
SsMADS1	willow_GLEAN_10012476	MIKCc	chr01	101	20	50	209	24.00	8.53	6
SsMADS2	willow_GLEAN_10012473	MIKCc	chr01	97	6	6,17	232	27.06	9.1	7
SsMADS3	willow_GLEAN_10014137	Mγ	chr01	46	-	-	401	43.52	7.05	0
SsMADS4	willow_GLEAN_10007397	Mα	chr01	47,48	-	-	530	60.23	6.67	8
SsMADS5	willow_GLEAN_10007399	Mα	chr01	47,48	-	-	212	24.34	6.22	0
SsMADS6	willow_GLEAN_10011253	Mβ	chr01	90	-	-	595	67.62	9.16	7
SsMADS7	willow_GLEAN_10022499	MIKCc	chr02	69	APETALA3	16	220	25.64	9.15	6
SsMADS8	willow_GLEAN_10020801	MIKCc	chr02	64	PISTILLATA	16	829	92.12	6.57	7
SsMADS9	willow_GLEAN_10020993	MIKCc	chr02	68	24	47	222	24.91	8.33	6
SsMADS10	willow_GLEAN_10021024	MIKCc	chr02	66	16	57	217	24.78	9.59	5
SsMADS11	willow_GLEAN_10011768	MIKCc	chr02	71	14	50	165	18.94	9.35	4
SsMADS12	willow_GLEAN_10020216	Mβ	chr02	67,102	-	-	405	45.69	7.57	0
SsMADS13	willow_GLEAN_10025520	MIKCc	chr03	94	14	50	287	32.58	10.07	5
SsMADS14	willow_GLEAN_10008017	MIKCc	chr03	95	2,9	7/45,8/24	245	27.96	8.58	7
SsMADS15	willow_GLEAN_10008015	MIKCc	chr03	35,26	-	6,17	263	29.40	9.31	4
SsMADS16	willow_GLEAN_10008014	MIKCc	chr03	35,26	-	6,17	218	24.65	7.83	6
SsMADS17	willow_GLEAN_10017246	MIKCc	chr04	25	AGAMOUS	58	350	39.19	9.3	8
SsMADS18	willow_GLEAN_10011967	Mα	chr04	21	-	-	194	21.74	9.08	0
SsMADS19	willow_GLEAN_10011966	Mα	chr04	27	29	-	178	20.10	9.96	0
SsMADS20	willow_GLEAN_10009082	MIKCc	chr05	53	-	29	219	25.34	8.54	4
SsMADS21	willow_GLEAN_10027002	MIKCc	chr06	43	15	57	250	28.08	8.65	7
SsMADS22	willow_GLEAN_10025994	Mγ	chr06	44	48	-	469	51.05	5.84	0
SsMADS23	willow_GLEAN_10026418	Mα	chr06	12,42	-	-	374	40.67	4.44	0
SsMADS24	willow_GLEAN_10012682	MIKCc	chr07	49	APETALA3	16	229	26.62	8.84	6
SsMADS25	willow_GLEAN_10007501	MIKCc	chr07	53	90	29	233	27.19	7.71	5
SsMADS26	willow_GLEAN_10007031	MIKC*	chr07	52	104	63	364	41.19	5.61	10
SsMADS27	willow_GLEAN_10014009	Mα	chr07	6	43	-	254	28.19	9.17	1
SsMADS28	willow_GLEAN_10014039	MIKC*	chr07	51	-	-	169	19.01	9.3	4
SsMADS29	willow_GLEAN_10024615	Mβ	chr08	84	-	-	202	22.90	6	0
SsMADS30	willow_GLEAN_10024753	Mα	chr08	17	-	-	197	22.70	9.36	0
SsMADS31	willow_GLEAN_10025082	MIKC*	chr08	85	30	68	357	39.79	6.95	9
SsMADS32	willow_GLEAN_10025158	MIKCc	chr08	87,95	2,9	7/45,8/24	241	27.62	5.65	7
SsMADS33	willow_GLEAN_10025159	MIKCc	chr08	86	7	15	212	24.53	8.48	5
SsMADS34	willow_GLEAN_10008129	MIKC*	chr09	57	-	-	80	9.23	10.33	1
SsMADS35	willow_GLEAN_10022978	Mα	chr09	19	-	-	205	23.07	5.29	0
SsMADS36	willow_GLEAN_10023049	MIKCc	chr09	15	15	29	259	29.39	8.81	7
SsMADS37	willow_GLEAN_10024397	MIKCc	chr09	89,66	44	57,61	263	30.14	9.39	6
SsMADS38	willow_GLEAN_10024365	Mα	chr09	18	43	-	416	46.75	9.62	2

SsMADS39	willow_GLEAN_10021705	Mα	chr10	29,7	-	-	203	23.09	5.25	0
SsMADS40	willow_GLEAN_10013611	MIKC*	chr10	85	30	68	894	98.51	6.62	13
SsMADS41	willow_GLEAN_10019310	Mβ	chr10	2	-	-	342	37.50	8.32	1
SsMADS42	willow_GLEAN_10004380	Mβ	chr10	1	-	-	201	22.46	5.02	0
SsMADS43	willow_GLEAN_10005930	MIKCc	chr11	41	AGAMOUS	3	227	25.81	9.62	5
SsMADS44	willow_GLEAN_10013792	MIKCc	chr12	103	-	34	135	15.72	9.47	3
SsMADS45	willow_GLEAN_10006110	MIKCc	chr13	103	-	34	232	26.73	8.84	5
SsMADS46	willow_GLEAN_10016051	MIKCc	chr14	82	6	7,16	218	25.40	9.85	6
SsMADS47	willow_GLEAN_10016052	MIKCc	chr14	83	20	50	218	25.38	9.55	6
SsMADS48	willow_GLEAN_10004716	Mβ	chr15	60	-	-	220	25.26	6.85	0
SsMADS49	willow_GLEAN_10009701	MIKCc	chr15	-	20	50	266	31.05	8.98	7
SsMADS50	willow_GLEAN_10023443	MIKCc	chr16	95	2,9	7/45,8/24	267	30.54	6.26	8
SsMADS51	willow_GLEAN_10003749	MIKCc	chr16	94	14	50	255	28.99	9.34	7
SsMADS52	willow_GLEAN_10002958	Mβ	chr16	20	-	-	265	30.53	5.37	0
SsMADS53	willow_GLEAN_10003926	MIKCc	chr17	23	29	7/45,8/24	245	28.17	8.27	7
SsMADS54	willow_GLEAN_10003927	MIKCc	chr17	14,26	8	14,15	238	27.54	9.18	6
SsMADS55	willow_GLEAN_10006611	Mα	chr18	-	-	-	310	33.64	4.74	0
SsMADS56	willow_GLEAN_10013302	MIKCc	chr19	72,31	12	26	321	36.31	8.47	4
SsMADS57	willow_GLEAN_10001835	MIKC*	N/A	45	-	-	82	9.51	9.9	1
SsMADS58	willow_GLEAN_10001302	MIKCc	N/A	31	12	26	156	17.88	9.1	3
SsMADS59	willow_GLEAN_10001292	Mγ	N/A	34	80	-	235	26.81	9.27	0
SsMADS60	willow_GLEAN_10000968	Mγ	N/A	-	-	-	158	18.14	5.99	0

Chr, chromosome numbers

N/A, not available

"-", not detected

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Table 2 (on next page)

Number of MADS-box genes in different species.

1 **Table 2.** Number of MADS-box genes in different species.

2

Phylum	Class	Order	Family	Species	Genome Size	Total	Type I	Type II
Angiosperms	Eudicots	Malpighiales	Salicaceae	*Salix Suchowensis*	425Mb	60	22	38
				Populus trichocarpa	480Mb	103	41	64
		Rosales	Rosaceae	*Malus domestica*	742Mb	146	64	82
		Fabales	Fabaceae	*Glycine max*	1100Mb	106	34	72
	Monocots	Poales	Poaceae	*Zea mays*	2300Mb	75	32	43
				Oryza sativa	466Mb	75	28	47
				Brachypodium distachyon	260Mb	57	18	39
Gymnosperm	Ginkgoopsida	Ginkgoales	Ginkgoaceae	*Ginkgo biloba*	10.61Gb	26	/	/
	Pinopsida	Pinales	Pinaceae	*Pinus taeda*	22Gb	11	/	/
				Picea sitchensis	/	17	1	16
	Cycadopsida	Cycadales	Cycadaceae	*Cycas elongata*	/	12	2	12

3

4 "/", not available