Gene Confering Abiotic Stress Tolerance in Plants

Zhong-Hui Cao1,2,3,*, Shi-Zhong Zhang1,2,3,†, Rong-Kai Wang1,2,3, Rui-Fen Zhang1,2,3, Yu-Jin Hao1,2,3,‡

1 State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai-An, Shandong, China, 2 MOA Key Laboratory of Horticultural Crop Biology and Germplasm Innovation, Shandong Agricultural University, Tai-An, Shandong, China, 3 College of Horticulture Science and Engineering, Shandong Agricultural University, Tai-An, Shandong, China

Abstract

The MYB proteins comprise one of the largest families of transcription factors (TFs) in plants. Although several MYB genes have been characterized to play roles in secondary metabolism, the MYB family has not yet been identified in apple. In this study, 229 apple MYB genes were identified through a genome-wide analysis and divided into 45 subgroups. A computational analysis was conducted using the apple genomic database to yield a complete overview of the MYB family, including the intron-exon organizations, the sequence features of the MYB DNA-binding domains, the carboxy-terminal motifs, and the chromosomal locations. Subsequently, the expression of 18 MYB genes, including 12 were chosen from stress-related subgroups, while another 6 ones from other subgroups, in response to various abiotic stresses was examined. It was found that several of these MYB genes, particularly MdoMYB121, were induced by multiple stresses. The MdoMYB121 was then further functionally characterized. Its predicted protein was found to be localized in the nucleus. A transgenic analysis indicated that the overexpression of the MdoMYB121 gene remarkably enhanced the tolerance to high salinity, drought, and cold stresses in transgenic tomato and apple plants. Our results indicate that the MYB genes are highly conserved in plant species and that MdoMYB121 can be used as a target gene in genetic engineering approaches to improve the tolerance of plants to multiple abiotic stresses.

Introduction

MYB TFs are widely distributed in all eukaryotic organisms, and these proteins comprise a large family of plant TFs, the members of which perform a variety of functions in plant biological processes [1], [2]. The MYB TFs are classified into three subgroups according to their MYB domain arrangement: R1R2R3, R2R3, and MYB-related (which contain a single MYB-like domain to recognize the major groove of DNA) [3], [4]. The major MYB TFs are the R2R3 MYB types, which have a modular structure with an N-terminal DNA-binding domain (the MYB domain) and an activation or repression domain that is usually located at the C terminus [3]. An increasing number of plant MYB TF members have been identified and characterized in numerous plant species based on the highly conserved DNA-binding domains in Arabidopsis [126 R2R3 MYB, five 3RMYB, and one 4R-like members], Populus trichocarpa [192 R2R3 MYB and five 3RMYB members], Cucumis sativus [55 R2R3 MYB members], and soybean [244 R2R3 MYB, six R1R2R3 MYB, and two 4R-like MYB members] [1], [5–7]. The 126 members of the R2R3 MYB family in Arabidopsis thaliana have been divided into subgroups [3]. New R2R3 MYB subgroups were identified according to comparative phylogenetic studies in other plant species such as in poplar and grapevine for which there are no representatives in Arabidopsis thaliana [3]. The expansion of the R2R3 MYB gene family in plants fits well with the observation that many R2R3 MYB TFs play important roles in plant-specific processes [3], [8]. In the past decade, the MYB genes, especially R2R3 M1Is, have been reported to be involved in diverse plant processes, including hormone signaling, cell cycle control, secondary metabolism, cellular morphogenesis, and meristem formation [8], [9]. For example, the overexpression of AtMYB75/PAP1 and AtMYB90/ PAP2 results in the accumulation of anthocyanins in Arabidopsis [10], [11]. The R2R3 MYB proteins of subgroup 12 regulate glucosinolate biosynthesis, whereas AtMYB28, AtMYB29, and AtMYB76 regulate the biosynthesis of aliphatic glucosinolates in aerial tissues [12], [13]. PhMYB1, AmMYB8L2, and AmMYB6 are able to induce changes in the epidermal cell shape [14]. In addition, some R2R3 MYB members have also been shown to regulate plant responses to biotic and abiotic stress conditions [3]. For example, AtMYB96 acts through the ABA signaling pathway to induce pathogen resistance by promoting salicylic...
acid biosynthesis and thus regulating stomatal movement, drought tolerance, and disease resistance in Arabidopsis [15], [16]. An R2R3-type MYB TF that is encoded by Hos10 is required for cold acclimation in Arabidopsis plants [17]. The overexpression of the rice OsMYB4 gene increases the chilling and freezing tolerance in transgenic Arabidopsis thaliana plants [10]. The ectopic expression of the apple MdMYB10 gene in Arabidopsis enhances tolerance to osmotic stress [19]. Transgenic Arabidopsis plants that contain FLP and AB1MYB89 elevate their tolerance to abiotic stress by restricting the divisions that occur late in the stomatal cell lineage [20].

Large numbers of MYB proteins have been characterized through genetic approaches. There has been much effort devoted to the identification of R2R3 MYB in response to abiotic stresses in the model plants Arabidopsis, rice, and other species. Although several MYB genes with roles in secondary metabolism have been characterized in apple and other fruit trees [21], no previous study of this family has been conducted in apple. The draft genome sequence of the apple has been released (http://genomics.iasma.it/) [22], [23]. In this study, a genome-wide analysis was conducted based on the conserved DNA-binding domain to identify the MdMYB gene models, the phylogenetic relationship of the MdoMYB proteins with other MYBs from different plant species, the genomic structure, the chromosome localization and other structural features. The expression patterns of twenty members in response to abiotic stresses were analyzed through real-time quantitative RT-PCR. Subsequently, we isolated an R2R3 MYB gene MdoMYB121 due to its stress-induced expression and high similarity to other stress-related R2R3 MYBs from other species. Furthermore, its function was characterized in transgenic tomato and apple plants. Finally, a further exploration of the value of the R2R3 MYB function and the potential uses of the MdoMYB121 gene in the improvement of the resistance of transgenic plants to abiotic stresses are discussed.

Results

Identification of Apple MdoMYB Genes

To identify the MYB-encoding genes in the apple genome, all known Arabidopsis MYB gene sequences were used as queries in multiple database searches against the proteome and genome files that were downloaded from the Apple GFDB database (Apple Gene Function and Gene Family Database: http://www.applegen.org/) and GDR database (Genome Database for Rosaceae: http://www.rosaceae.org/). Approximately 300 sequences that contain MYB or MYB-like repeat genes were identified in the apple genome. To confirm the putative MYB gene models that were identified, all of the genes derived from the selected MdoMYB candidate genes were examined using the domain analysis programs Pfam and SMART with the default cutoff parameters. In addition, we analyzed the domains of all of the apple peptide sequences through an HMM (Hidden Markov Model) analysis using Pfam. As a result, 222 typical R2R3 MYB proteins, five R1R2R3 MYB proteins, and two 4R-like MYB proteins were confirmed from the original data. This number is 1.7-fold higher than that found in Arabidopsis thaliana. To distinguish the remaining MYBs, we provisionally named them MdoMYB1 through MdoMYB229 based on their location on the chromosome, which was identified from the apple genome browser (Table S1). Among these MdoMYBs, the predicted proteins MdoMYB39, MdoMYB43, MdoMYB92, MdoMYB138 and MdoMYB190 belong to the R1R2R3 MYB family. In addition, MdoMYB175 and MdoMYB176 belong to the 4R-like MYB protein family. Additionally, we include the gene identifier, the genomic position, the pI (isoelectric point), the ORF length, the amino acid length, and the synonym in Table S1. As shown in this table, all of the identified MYB gene models encode proteins ranging from 126 (MdoMYB17) to 1710 (MdoMYB112) amino acids, and have a protein pI ranging from 4.61 (MdoMYB34) to 10.29 (MdoMYB161).

Phylogenetic and Intron-Exon Structure Analysis of the Apple MYB Gene Family

To evaluate the evolutionary relationships within the MYB gene family, we performed a combined phylogenetic analysis of the Arabidopsis (132 members) and apple (229 members) MYB proteins using the NJ (neighbor-joining) method of the MEGA5 program (Figure S1). Furthermore, the peach and pear MYB families, which have 121 and 167 members, respectively, were also combined into the phylogenetic tree. The result showed high similarity among the Rosaceae MYBs (Figure S2). Based on sequence similarity and topology, we subdivided the apple MYB protein family into 45 subgroups, which were designated S1 through S25, H1 through H15, and performed a bootstrap analysis with 1,000 replicates for support (Figure S1A). As shown in Figure S1, all R2R3 MYB subgroups clustered separately from R1R2R3 and 4R MYB members. In our subgroup classification of the MYB proteins, we also took into account the results of Kranz et al. [4], Yanhui et al. [5], and Dubos et al. [3]. In addition, the functions of known AtMYBs are annotated (Figure S1B and references are shown in Text S2). Subgroups such as S1, S4, S11, S14, S20, S18, S21, S22, and H17 contained Arabidopsis MYB TFs which are known to be involved in the responses to abiotic stresses (Figure S1B).

We found that it was common to find that two or more MdoMYBs appeared to be putative orthologs of a single protein in Arabidopsis; for example, the phylogeny of subgroup H7 included only one AtMYB and five MdoMYBs. In contrast, six and three AtMYBs were included in subgroup S12 and S19, respectively. In addition, the phylogeny of subgroup S14 has the largest numbers of MYBs, including 14 MdoMYBs and 6 AtMYBs; subgroup S10, S19, H2, H8, H13, H14, H15, and H18 included the least number of MYBs.

In addition, the results of the intron-exon structure identification of the MdoMYB gene models are shown in Figure S3. To determine the numbers and positions of the exons and introns within each apple MYB gene model, we compared the full-length cDNA sequences with the corresponding genomic DNA sequences of the MIB distributed by introns. Only 7% of the MYBs had no introns in the coding region, whereas the remaining genes had up to 20 introns (MdoMYB190) based on their relative positions and phases.

Sequence Features, Chromosomal Distribution and Duplication Events of the Apple MYB Gene Models

To investigate the DNA-binding domains of the apple MYB proteins, logos were generated using WebLogo, which is designed to show the conservation at a particular positions within a multiple sequence alignment [24]. The results showed that the R2 and R3 repeats of MdoMYBs contain conserved amino acids, especially characteristic Trp residues (Figure 1A–B). Among these, the R2 repeat contains three conserved Trp residues. The R3 repeat also exhibited a high conservation of the second and third Trp residues, whereas the first Trp was generally replaced by Phe or Ile. Subsequently, MEME software was used to identify the structural similarities of C-terminal motif. It was found that, among 45 subgroups analyzed, 27 ones possessed one to four
identical C-terminal motives in each subgroup, while the other 18 did not at all (Figure S1C).

The information concerning the expansion events of the MYB gene family in apple remain unclear so far. Therefore, the chromosomal locations of the MdoMYB genes were retrieved from the
apple genome data that were downloaded from the GDR database. The analyses of the genome chromosomal locations revealed that the apple MIB genes are distributed on all chromosomes. Although each of the 17 apple chromosomes contained MIBs, the distribution appeared to be uneven (Figure 1C). Seven gene models (MdoMYB223, 224, 225, 226, 227, 228, and 229) could not be conclusively mapped to any chromosome. The gene density per chromosome ranged from 4.1% to 9.9%; the highest density was observed on chromosome 14 (22 members), and the lowest density was found on chromosome 2 (nine members). In general, the middle part of the chromosomes exhibited a low density of MIB genes, whereas the ends exhibited a relatively high density. Also, large-scale segmental duplication events were investigated. It was found multiple pairs linked each of at least 15 potential chromosomal/segmental duplications (Figure 1C, pairs of bars with numbers 1 through 15 in the blue areas), such as the large sections on chromosomes 3 and 11.

**Expression Profiles for 20 Mdr2R3 MYB Genes in Response to Abiotic Stresses**

Mounting evidence suggests that the R2R3 MYB genes play important roles in abiotic stress tolerance in various plant species. To examine if the expression of apple R2R3 MYB genes is induced by abiotic stresses including NaCl, ABA, PEG, and cold treatments, 12 apple R2R3 MYB gene models (MdoMYB54, 67, 97, 107, 146, 148, 155, 185, 197, 199, 206, and 222, Figure 2A) were chosen from stress-related subgroups, while another 6 ones (MdoMYB11, 22, 109, 121, 133, 136, Figure 2B) from other subgroups. These gene models represented really genes as demonstrated by EST blast through the GenBank database and sequencing of the corresponding RT-PCR production (Text S1).

Subsequently, real-time RT-PCRs were performed to analyze their expressions in response to four treatments, i.e. NaCl, ABA, PEG, and cold (Figure 2, and primers are listed in Table S2). The results showed that the transcript levels of MdoMYB22, 121, 146, 148, 155, and 206 increased in response to the four treatments. The levels of MdoMYB11, 67, 109, 197, and 222 were enhanced in response to only two or three of the treatments. The expressions of MdoMYB133 and MdoMYB185 were induced by ABA, and PEG, respectively, but inhibited by the other treatments. In contrast, the transcription levels of MdoMYB54, 97, 107, 136, and 199 were downregulated by these treatments.

**Seed Germination and Seedling Growth are Insensitive to High Salinity in MdoMYB121 Transgenic Tomato**

MdoMYB121 (Genebank accession number KC834015) was subgrouped into H7 R2R3 MYB subgroup which does not contain known stress-related MIB gene (Figure S1). However, its expression was remarkably induced by the four abiotic stress treatments imposed (Figure S4). The phylogenetic tree further demonstrated that MdoMYB121 exhibited similarity to different extents to abiotic stress-related MYBs from other species (Figure S3). Also, MdoMYB121-GFP fusion protein is subcellularly localized in nucleus, just like other transcription factors (Figure S6). Therefore, MdoMYB121 was chosen for functional characterization. MdoMYB121 gene was isolated from apple cDNA template and then genetically transformed into tomato. Three lines, named OE-1, OE-2, and OE-4, were used for further investigation (Figure 3A). The MdoMYB121 transcripts showed high levels in the functional leaves of the three selected transgenic lines, but no transcripts were detected in the wild-type tomato using primers specific for MdoMYB121. Then, the seed germination and young seedling growth were examined to determine their response to high salinity. There was no apparent difference in the seed germination between the WT and the transgenic plants under normal growth conditions. Compared with the WT, the MdoMYB121 plants exhibited significantly higher seed germination in response to 30 mM NaCl (Figure 3B). For example, approximately 70% of the MdoMYB121-overexpressing seeds germinated at day 6 compared with 30% of the WT seeds.

Subsequently, the effects of the ectopic expression of MdoMYB121 on tomato seedlings under salt stress were determined. The transgenic and WT seedlings pre-germinated on MS medium and then transferred to media containing 200 mM and 300 mM NaCl. Under 200 mM NaCl, the transgenic seedlings formed longer roots and exhibited faster development of green cotyledons than the WT, although the growth of both the WT and the transgenic seedlings was significantly inhibited (Figure 3C). When the concentration of the NaCl was increased to 300 mM, the growth of the WT was completely inhibited, and the cotyledons could not form normally. However, the transgenic seedlings formed longer roots, and some of them developed green cotyledons and continued to grow (Figure 3D).

These results suggest that the overexpression of MdoMYB121 confers an increased tolerance to salt stress during seed germination and early seedling development.

**Ectopic Expression of MdoMYB121 Confers Enhanced Tolerance to Abiotic Stresses in Tomato**

We then tested the influence of the ectopic expression of MdoMYB121 on adult tomatoes exposed to salt, drought, and cold. All of the adult plants perform well, and there were no significant differences between the WT and the transgenic tomato plants under normal growth conditions (Figure 4A). The transgenic tomato plants grew well when irrigated with 300 mM NaCl solution for 12 days, whereas the WT plants grew poorly and exhibited chlorosis. After twenty days, the WT plants died. In contrast, the leaves of the transgenic plants turned yellow and wilted significantly, but continued to grow (Figure 4B).

To elucidate why transgenic plants are more tolerant to high salinity than the WT plants, the Na⁺ content and the Na⁺/K⁺ ratio in the functional leaves were measured under salt stress. The results show that the transgenic lines accumulated a lower Na⁺ content and a lower Na⁺/K⁺ ratio than the WT plants, which indicates that MdoMYB121 is involved in the regulation of the physiological balance between Na⁺ and K⁺ in response to salt stress (Figure 5A).

For the dehydration tolerance assay, transgenic MdoMYB121 and WT tomato plants were withheld from water for 15 days and then re-watered for 3 days (Figure 4C). After 10 days, the transgenic plants grew well under normal growth, whereas the WT plants wilted. After 15 days, almost all of the WT had died, and all transgenic plants were adversely wilted. However, the growth of the transgenic plants recovered after re-watering, whereas the WT plants did not.

To estimate the water loss under drought conditions, the relative water content (RWC) in WT and transgenic MdoMYB121 plants were measured. The results indicate that the transgenic lines exhibit a greater potential to maintain water than the WT plants (Figure 5B), which indicates that the overexpression of MdoMYB121 enhances drought tolerance at least partially by reducing the water loss.

Tomato plants were exposed to 4°C to test whether the MdoMYB121 ectopic expression enhances cold tolerance. After 4 days of the cold treatment, the WT plants were adversely affected and wilted, but the transgenic plants appeared normal in
morphology (Figure 4D). Most of the WT plants died after 13 days, whereas the transgenic lines were significantly wilted but performed better. Subsequently, the transgenic plants and WT were transferred to normal conditions for a 3-day recovery period. The results show that the growth of the transgenic plants completely recovered, although the leaves were shrivelled and yellow, but the WT plants died.

To investigate the physiological mechanisms underlying the function of MdoMYB121 in response to abiotic stresses, the contents of the membrane damage and the intracellular oxidative stress responsive index, such as the relative electrolyte leakage and the MDA and proline levels, were measured [25–27]. The results show that the transgenic MdoMYB121 tomato plants exhibit lower levels of electrolyte leakage and MDA and an elevation in the content of the osmoprotectant proline in response to salt, drought, and cold stresses (Figure 5C). We hypothesize that MdoMYB121 reduces the membrane damage and promotes the accumulation of protective compounds against environmental stress.

MdoMYB121 Overexpression Enhances Tolerance to Abiotic Stresses in Transgenic Apple Rooted Plantlets

The performance of the MdoMYB121 transgenic tomato plants under abiotic stresses indicate that this gene might also operate in apple. To confirm that MdoMYB121 really confers tolerance to salt, drought, and cold stress in apple, transgenic apple plants carrying MdoMYB121 were produced. Three transgenic lines of each MdoMYB121 (T1, T2, and T4) were chosen for further

---

Figure 2. Expression analysis of 18 Mdr2r3 MYB genes under abiotic stress treatments. The expression pattern of 18 Mdr2r3 MYB genes in response to NaCl, ABA, PEG, and cold treatments at 2 h, 12 were chosen from stress-related subgroups (A) and 6 ones from other subgroups (B). MdACTIN was used as an internal standard.

doi:10.1371/journal.pone.0069955.g002
investigation (Figure 6A). In the tolerance assays, six-month-old transgenic self-rooted plantlets were used.

For the analysis of salt tolerance, the transgenic and WT plantlets were irrigated with 300 mM NaCl solution for 16 days. After 14 days, the leaf color of the MdoMYB121 lines were normal, whereas the WT controls were discolored, which indicates that the leaf necrotic damage of the WT control was more serious than that found in the transgenic lines (Figure 6B). After 16 days, almost all of the WT plants died, whereas the transgenic plants performed better, although some leaves, especially the young leaves, were discolored. These results suggest that the MdoMYB121 overexpression confers enhanced salt tolerance in the transgenic apple plants.

For the drought tolerance assay, the WT and transgenic rooted plantlets were deprived of water for 15 days and then re-watered for 3 days. The results show that the WT plants wilted after being deprived of water for 12 days and that the transgenic lines performed well (Figure 6C). After 15 days, the WT plants exhibited a noticeable loss of color, brittle phenotypes, and even death, whereas the transgenic apple plants were inhibited to a lesser extent. After relief of the water deficit stress for 3 days, the transgenic lines demonstrated a recovery phenotype, but the WT plants died. These results demonstrate that the MdoMYB121 transgenic apple plants exhibit noticeably enhanced tolerance to drought stress treatment.

The cold stress tolerance of the MdoMYB121 apple plants was then tested. Compared with the MdoMYB121 plants, the WT plants had a significantly wilted phenotype in the leaves after exposure to 4°C for 14 days (Figure 6D). After 16 days, the leaves of all of the WT and transgenic apple plants turned red, but the WT plants exhibited more serious necrotic damage compared with the transgenic lines. Finally, the WT and transgenic apples were returned to normal conditions at 22°C for an additional 25 days. We observed that the growth of all of the plants recovered, but the transgenic plants grew better compared with the WT. Therefore, MdoMYB121 overexpression enhances cold tolerance in transgenic apple plants.

Discussion

The MYB transcription family is large, functionally diverse, and represented in all eukaryotes. It is one of the most abundant transcription factor families in plants and has been identified in Arabidopsis, rice, maize, soybean, and grape [1], [7], [28], [29]. In this study, we identified 229 apple MYB gene models from the apple genome sequence, which contribute to a large plant MYB TF family. Furthermore, the overexpression of an R2R3 MYB gene MdoMYB121 enhances tolerance to abiotic stresses in both tomato and apple plants.

Compared with MYB proteins in various plant species, the MYBs that tended to cluster together were usually from the same lineage, which indicates recent common evolutionary origins. In this study, putative orthologs of a single AtMYB protein with two or more apple MYBs were found, which indicates that the apple MYBs experienced duplications after their divergence from Arabidopsis. The occurrence of gene duplication throughout plant evolution has long been recognized and contributes to the expansion of the apple MYB genes and the establishment of the new gene functions. In this study, MdoMYB121 belongs to the subgroup H7 and exhibits similarity to AtMYB5, which has been characterized to have functions in seed coat differentiation and is partially redundant with AtMYB125 in the regulation of tannin [3]. Therefore, the response to abiotic stresses of MdoMYB121 expands the function of this subgroup, and AtMYB5 may be related to stress induced responses.

In general, the gene functions of a subgroup appear to be similar. For example, the AtMYB in subgroups S6 and S15 are needed for anthocyanin biosynthesis and the regulation of trichome development in Arabidopsis, respectively. Therefore, not all members of the same subgroup perform an absolutely conserved function. In subgroup S4, AtMYB32 is involved in the
normal pollen development in *Arabidopsis thaliana* [3], whereas *AtMYB8* is required for freezing tolerance and its loss of function results in hypersensitivity to NaCl and increases the transcripts of stress-related genes [30].

Remarkably, the genes in the same group generally exhibit the same intron pattern, and the position of the intron is almost completely conserved within most subgroups [1]. Additionally, the first two exons of the modal lengths are very similar and highly conserved [31]. This finding tests the reliability of our phylogenetic analysis and constitutes an independent criterion. However, in our study, the members of *MdoMYBs* within the same subgroup did not always show similar intron-exon structures and formed a complex exon/intron organization in the entire ORF.

In general, MYB proteins are characterized by a highly conserved DNA-binding domain in the N-terminus [1], [3], [4], [32]. This domain mainly consists of up to four imperfect repeats (R) and contains three helices of each repeat, the second and third of which form a helix-turn-helix structure when bound to DNA [3], [4], [7]. There are R2 and R3 repeats among the 222 MdR2R3 MYB proteins. Three highly conserved Trp residues were generally contained in the R2 repeat, whereas the first Trp residue in the R3 repeat exhibits variability. The substitution at this first Trp residue may be responsible for the recognition of novel target genes and/or may significantly impair the DNA-binding activity [33]. In contrast, the other regions, especially the C-terminus, of the R2R3 MYB proteins are highly variable [3], [7]. However, the conserved C-terminal motifs of MYB proteins were identified in soybean, *Arabidopsis*, and rice [1], [4], [34]. In this study, it was found that some apple MYB proteins have conserved C-terminal motifs, suggesting that they maybe have similar functions. In addition, some MYB proteins have been identified from different species. They share an identical C-terminal W/Y-MDDIW motif which is important for their trans-activation activity [35]. Based on the conserved N-terminus and the transcriptional activation or repression in the C-terminal domain of the MYB TFs, these proteins play important roles in multiple plant physiological processes, such as tolerance to abiotic stresses.

During the evolution of plants, gene duplication events have long occurred. These contribute to the establishment of new gene
functions and underline the origins of evolutionary novelty [36], [37]. Relatively recent genome-wide duplication (GWD) events and the expansion of gene families in the Pyreae resulted in the transition from nine ancestral chromosomes to 17 chromosomes [22]. In this study, it was found that 15 potential chromosomal/segmental duplications of multiple pairs are associated with genome-wide duplication in the apple genome, as has been reported by Velasco et al. [22], who supported the hypothesis that the large-scale expansion of the R2R3 MYB family in the apple genome evolved from putative duplication events. Furthermore, there exist several types of segment pairs during chromosomal duplication events. For example, they were classified into α, β, and γ type in Arabidopsis according to the relative time of duplication [38]. It has been previously reported that subgroup S12, which is responsible for regulate aliphatic or indolic glucosinolate biosynthesis in Arabidopsis, comes from a specific β-type duplication in the order Brassicales to adapt herbivory [5], [39], [40]. However, there is no apple, rice and grape MYB gene models grouped into S12, probably because β-type duplication does not occur in these species [5], [31]. In addition, many fragments have not yet been assigned to a particular chromosome on the draft genome sequences [22], and this may the reason why seven genes could not be conclusively mapped to any chromosome.

It has been well established that the MYB s play important roles in various life processes [2], [3], [15]. In fact, many MYB genes in a wide range of plant species, such as Arabidopsis, rice, and wheat, are involved in the response to abiotic stresses [3], [15], [41]. In Arabidopsis, the overexpression of AtMYB44 and OsMYB4 enhances tolerance to abiotic stresses [2], [18]. In wheat (Triticum aestivum), TaMYB33 exhibited high similarity with other stress-related MYBs. Its transcription is induced by abiotic stresses, and its overexpression increases the tolerance of the plant to abiotic stress [41]. In grape, VvMYB60 is identified to an ortholog of AtMYB60 according to a bioinformatic- genomic analysis, and is characterized to be involved in stomatal regulation and thus in abiotic stress responses [42]. However, little is known about the specific roles of MYB genes in apple in response to abiotic stresses; in fact, just a few of MdMYB genes is found to be involved in stress tolerance, such as MdMYB10 [19]. Our genome-wide analysis and expression profiling of apple MYB TFs in response to multiple abiotic stresses provide a baseline for their further functional characterization with stress tolerance. Accordingly, MdoMYB121 was chosen to further investigate whether it plays a role in the responses of plants to abiotic stresses, as many R2R3 MYB TFs do in other plant species [3]. It was found that MdoMYB121 overexpression enhances tolerance to high salinity, drought, and cold in transgenic tomato and apple plants.

Plant salt tolerance is a complex trait. Multiple physiological and biochemical mechanisms are involved to maintain a high cytosolic K⁺ uptake, prevent excess Na⁺ accumulation in the plant symplast, and/or maintain desirable K⁺/Na⁺ ratios in the cytosol [43], [44]. Thellungiella halophila is a salt-tolerant relative of...
Arabidopsis thaliana. This plant exhibits extreme tolerance to high salinity through the support of K⁺/Na⁺ homeostasis in the root ion-channel [45]. A mutation in the Salt Overly Sensitive 2 (SOS2) gene causes an Na⁺ and K⁺ imbalance that renders the mutant plants more sensitive to growth inhibition in high Na⁺ and low K⁺ environments [46]. Additionally, the transporter AtHKT1 controls Na⁺ entry and high affinity K⁺ uptake in the leaves of Arabidopsis to determine its tolerance to salt stress [47]. In this study, MdoMYB121 transgenic tomato plants accumulated less Na⁺ and maintained a lower Na⁺/K⁺ ratio under high salinity than the WT control plants, which shows that this gene enhances stress tolerance. The result suggests that MdoMYB121 is involved directly or indirectly in the regulation of Na⁺ and K⁺ homeostasis in plant cells under salt stress.

During dehydration periods, plants accumulate the stress-response hormone abscisic acid (ABA), which induces the rapid closing of stomata to prevent water loss by transpiration [48]. The R2R3 MYB proteins FOUR LIP (FLP) and MYB88 restrict divisions late in the stomatal cell lineage in Arabidopsis [49]. The flp-1 myb88 double mutant is much more susceptible to drought and shows increased rates of water loss [20]. The overexpression of AtMYB61 can minimize the water loss in response to drought stress and might be employed as a strategy for the growth of crop plants in arid regions [50]. Transgenic plants overexpressing OsMYB3R-2 exhibit a slower water loss in detached rosette leaves compared with WT, which indicates that this gene enhances drought tolerance [51]. In this study, MdoMYB121 transgenic lines maintained a higher leaf RWC and therefore exhibited a higher drought tolerance than WT controls in response to drought stress.

In addition, abiotic stresses result in membrane damage, as indicated by the MDA concentration and the electrolyte leakage. In parallel, proline is accumulated to protect the membrane integrity [27], [52–54]. In previous reports, an elevated tolerance at any given time because it closely reflects the balance between the water supply and the transpiration rate [48]. The R2R3 MYB proteins FOUR LIP (FLP) and MYB88 restrict divisions late in the stomatal cell lineage in Arabidopsis [49]. The flp-1 myb88 double mutant is much more susceptible to drought and shows increased rates of water loss [20]. The overexpression of AtMYB61 can minimize the water loss in response to drought stress and might be employed as a strategy for the growth of crop plants in arid regions [50]. Transgenic plants overexpressing OsMYB3R-2 exhibit a slower water loss in detached rosette leaves compared with WT, which indicates that this gene enhances drought tolerance [51]. In this study, MdoMYB121 transgenic lines maintained a higher leaf RWC and therefore exhibited a higher drought tolerance than WT controls in response to drought stress.

Figure 6. MdoMYB121 overexpression enhances tolerance to abiotic stresses in apple plantlets. (A) Expression levels of MdoMYB121 in independent transgenic apple lines. (B–D) Tolerance of transgenic rooting apple plantlets for 300 mM NaCl for 16 d, dehydration for 15 d with a 3 d recovery and 4 °C for 16 d with a 25 d recovery. doi:10.1371/journal.pone.0069955.g006
to osmotic and freezing stresses is accompanied by a lower MDA content and a higher proline accumulation in *MdoMYB10* and *OsMYB4* transgenic plants, respectively [10], [19]. In addition, the *AtMYB2* transgenic *Arabidopsis* exhibits a higher tolerance to osmotic stress, as measured by the electrolyte leakage from cells [55]. In this study, transgenic *MdoMYB121* plants produced more proline and less MDA and exhibited less electrolyte leakage than the control plants, which indicates that these plants exhibited enhanced tolerance to multiple stresses.

Taken together, our results indicate that the *MYB* genes are highly conserved among different plant species. The *in silico* analysis performed provides a valuable method for further exploration of the functions of the *R2R3 MYB* genes in apple. As a result, *MdoMYB121* was functionally characterized as a target gene for genetic engineering approaches to improve the tolerance of fruit trees and other crops to multiple abiotic stresses.

**Materials and Methods**

**Ethics Statement**

No specific permits were required for the described field studies. The location is not privately-owned or protected in any way, and the field studies did not involve endangered or protected species.

**The Identification of MYB Genes in Apple**

Two approaches were used to identify the members of the *MdoMYB* gene family in apples. First, all known *Arabidopsis MYB* gene sequences were used as queries to perform multiple database searches against the proteome and genome files that were downloaded from the Apple GFD database (Apple Gene Function and Gene Family Database: http://www.applegene.org/) as well as the GDR database (Genome Database for Rosaceae: http://www.rosaceae.org/). Stand-alone versions of BLAST (Basic Local Alignment Search Tool: http://blast.ncbi.nlm.nih.gov/) which are available from the NCBI, were used with an e-value cutoff of 1e-003. All of the protein sequences deriving from the selected *MdoMYB* candidate genes were examined with the domain analysis programs Pfam (Protein family: http://pfam.genome.org/) and SMART (Simple Modular Architecture Research Tool: http://smart.embl-heidelberg.de/) with the default cutoff parameters. Second, we analyzed the domains of all of the apple peptide sequences using an HMM (Hidden Markov Model) analysis while searching Pfam. Then, we obtained the sequences by using the Pf00249 Pfam number, which contained a typical MYB DNA-binding domain, from the apple genome sequences by making use of a Perl-based script. Finally, all of the protein sequences were compared with known *MdoMYB* sequences by applying Clustal X (http://www.clustal.org/) to verify that the sequences were candidate *MdoMYBs*. The isoelectric points and protein molecular weights were obtained with the help of the proteomics and sequence analysis tools on the ExPaSy proteomics server (http://expasy.org/). The chromosomal locations were found in the GDR database by using a Perl-based program.

In the same way, we obtained 167 *Pyrus sativum* MYB genes according to the *Pyrus* genome (http://peargenome.njau.edu.cn/). Additionally, 121 *Pyrus persica* MYB genes were obtained from the Genome Database for Rosaceae (GDR).

**Sequence Alignment and Phylogenetic Analysis**

The MYB sequences were aligned using the Clustal X program with BLOSUM 30 as the protein weight matrix. The MUSCLE program (version 3.52) was also applied to perform multiple sequence alignments to confirm the Clustal X result. The phylogenetic trees for the *MdoMYB* protein sequences were constructed using the *NJ* (neighbor-joining) method of the MEGA5 program (http://www.megasoftware.net/) and the p-distance for the complete deletion option parameters. The reliability of the trees was tested using a bootstrapping method with 1,000 replicates. The images of the phylogenetic trees were drawn in MEGA5.

**The Chromosomal Location and Structure of the MdoMYB Genes**

The chromosomal locations and gene structures were retrieved from the apple genome data that were downloaded from the GDR database. The remaining genes were mapped to the chromosomes with MapDraw, and the gene structures of the *MdoMYB* genes were generated with the GSDS software (http://gsds.chi.pku.edu.cn/) [56].

**Protein Motif Identification**

Motifs were detected within each subgroup using MEME version 3.5.7 tool to identify conserved motifs shared among MYB proteins [57]. The following parameter settings were used: distributing of motifs, zero or one per sequence; maximum number of motifs to find, 50; minimum width of motif, six; maximum width of motif, 117 (to identify long R2R3 MYB domains); and motif must be present in all members within the same subfamily.

**Plant Materials and Treatments**

*In vitro* shoot cultures of apple ‘Gala’ were maintained on MS medium containing 0.5 mg L\(^{-1}\) 6-BA, 0.2 mg L\(^{-1}\) IAA, and 0.1 mg L\(^{-1}\) GA under long-day conditions (16-h light/8-h dark cycle) and then subcultured with a 4-week interval. Four-week-old shoot cultures were transferred to a root-inducing medium, i.e., MS medium containing 0.1 mg L\(^{-1}\) IAA to obtain self-rooted plantlets. The rooted plantlets were then transferred to pots of nursery soil for further investigation.

Approximately 4-week-old apple shoot cultures were transferred to MS medium containing 200 mM NaCl, 4°C low temperature, 10% PEG, or 100 μM ABA. These were sampled after 0, 2, 4, 6, 12, and 24 h for the analysis of the gene expression in response to the stress treatments. The 5’ UTR-specific primers for the real-time quantitative RT-PCR analysis are listed in Table S2.

**Construction of MdoMYB121 Overexpression Vector**

The total RNA was isolated from *in vitro* shoot cultures of ‘Gala’ using the RNA plant plus Reagent (Tiangen, Beijing, China). Two micrograms of the total RNA was used to synthesize the first-strand cDNA for *MdoMYB121* gene cloning (Takara, Dalian, China). To generate *MdoMYB121*-overexpressing plants, the full-length cDNA of *MdoMYB121* was digested with *Xba*I and *Sal*I and cloned into the pB121 vector, in which it was placed under the control of the *CAMV* 35S promoter. Then, *Agrobacterium tumefaciens* strain LBA4404 was transformed with the construct. The PCR products used for the constructs were amplified from the *MdoMYB121* cDNA using the following primers: 5’-GCGTCGCAGTTGACGCCTTTGAGTCATCCTTTGGATCATTA-3’ (forward) and 5’-GGTCGTACGGATCCGAATTCACTTCGGCTTTGATCATTA-3’ (reverse).

**Subcellular Localization of MdoMYB121 Protein**

The subcellular localization of *MdoMYB121* protein was determined as described by Li et al. [58]. The *MdoMYB121* ORF without a stop codon was obtained by RT-PCR using the following primers containing digestion sites (underlined): 5’-
Tomato Transformation and Stress Tolerance Assay in Transgenic Tomato

Tomato (Solanum lycopersicum L. cv. ‘SN1’) cotyledons were transformed with the Agrobacterium tumefaciens strain LBA4404 containing the MdoMYB121-overexpressing constructs as described by Zhang and Blumwald et al. [59]. Three-month-old tomato plants were exposed to 300 mM NaCl solution in a growth chamber for 20 days for the salt tolerance assay. Then, the tomato plants were deprived of water for 15 days and then re-watered for 3 days for the drought tolerance assay. The tomato plants were transferred to a 4°C illuminated incubator for 13 days and grown at 22°C for an additional 3 days for the cold tolerance test.

Apple Transformation and Stress Tolerance Assay in Transgenic Apples

Leaf explants of ‘Gala’ were transformed with the Agrobacterium tumefaciens strain LBA4404 containing the MdoMYB121-overexpressing constructs as described by Kotoda et al. [60]. The transgenic in vitro shoots were transferred to root-inducing medium, i.e., MS medium containing 0.1 mg/L IAA and selectively transferred to pots containing nursery soil after one month. After six months, the self-rooted plantlets were transferred to pots to test their stress tolerance. The pot-grown plantlets were deprived of water for 15 days and re-watered for 3 days for the drought tolerance assay. The pot-grown plantlets were irrigated with 300 mM NaCl solution twice a week for 16 days for the salt tolerance assay. The apple plantlets were exposed to a temperature of 4°C for 16 days and then returned to normal conditions at 22°C for an additional 25 days for the cold tolerance test. All of the experiments were performed in triplicate.

Measurement of Relative Water Content and Relative Electrolyte Leakage

The relative water content (RWC) under abiotic stresses was determined as indicated: RWC = (fresh weight - dry weight)/(rehydrated weight - dry weight). The leaf cell membranes damaged were determined under abiotic stresses according to the method described by Premachandra et al. [61] and were calculated as indicated: electrolyte leakage = [(1 - (1 - S1/S2))/(1 - C1/C2)], the conductivity measurements corresponding to abiotic stresses treated leaves (S1), boiled abiotic stresses treated leaves (S2), non-abiotic stresses treated leaves (C1), and boiled non-abiotic stresses treated leaves (C2).

Determination of Malondialdehyde (MDA) Level and Free Proline Analysis

The MDA level according to the method as described by Hodges et al. [62]. Tomato leaves were homogenized in 5 ml 10% trichloroacetic (TCA) and centrifuged at 12,000 rpm for 10 min. A volume of 2 ml of clear supernatant was added to 4 ml of 0.6% thiobarbituric acid (TBA, in 10% TCA) and incubated at 100°C in a water bath for 15 min. The reaction was cooled to room temperature, and the absorbance of the supernatant of MDA was measured at 450, 532, and 600 nm using a UV-vis spectrophotometer (UV-2450). The MDA contents (µmol g⁻¹ FW) were calculated with the following formula: 6.45 (OD₅₃₂-OD₆₀₀) - 0.56OD₅₃₂. The free proline content in leaves was determined by essentially following the reported methods of Bates et al. [63]. The leaves of tomato under multiple treatments were homogenized in 5 ml sulfoacetic acid (3%), and the homogeneous mixture was centrifuged at 12,000 rpm for 15 min at 4°C. The extract was then filtered through three 3 ml ninyhydrin and 2 ml glacial acid for 30 min at 100°C and cooled to 4°C in 30 min. The absorbance of the toluene layers was measured at 520 nm with a spectrophotometer (Shimadzu UV-2450, Kyoto, Japan). The proline concentration was determined using a calibration curve and expressed as µg proline g⁻¹ FW.

Na⁺ and K⁺ Contents

To measure the Na⁺ and K⁺ contents, the tomato leaves were weighed after drying for 24 h at 75°C and then digested with a 4:1 (v/v) mixture of nitric and perchloric acids. These materials were filtered, diluted with distilled water, and subjected to atomic absorption spectroscopy (Perkin Elmer AA300, CT, USA) to determine the Na⁺ and K⁺ concentrations.

Supporting Information

Figure S1 Phylogenetic relationships and subgroup designations in MYB proteins from apple and Arabidopsis. (A) The tree represents relationships among 229 MYB proteins from apple, 132 (123 R2R3 MYBs, five R1R2R3 MYBs, and one 4R MYB) from Arabidopsis. The unrooted phylogenetic tree was inferred using the neighbor-joining method of the MEGA5 program. The numbers on the branches represent bootstrap values with 1000 bootstrap replicates. Bootstrap values <50% are not shown in the phylogenetic tree. The proteins are clustered into 45 subgroups based on clades at least 50% bootstrap values, which are designated with a subgroup number (e.g., S1 or H1) and marked with different colors to facilitate subgroup identification. (B) The functions of AtMYB genes are annotated and references are shown in Text S2. (C) Subgroups sharing one to four motifs are highlighted with blue color. (TIF)

Figure S2 Phylogenetic relationships of MYB proteins from apple, Arabidopsis, peach and pear. In the annotation, ppa (121 members) and Pbr (167 members) MYBs are from peach and pear, respectively. The unrooted phylogenetic tree was constructed using the neighbor-joining method of the MEGA5 program with 1000 bootstrap replicates. Bootstrap values <50% are not shown in the phylogenetic tree. (TIF)

Figure S3 Intron-exon structures of apple MYB genes. All 229 gene intron-exon structures are described on the right. Exons and introns are indicated by green boxes and single lines, respectively. The unrooted phylogenetic tree of 229 proteins from apple (on the left) was inferred using the neighbor-joining method of the MEGA5 program with 1000 bootstrap replicates. (TIF)

Figure S4 Expression analysis of the MdoMYB121 gene under abiotic stress treatments. MdoMYB121 expression levels in response to salt, ABA, PEG and cold as revealed by qRT-PCR. (TIF)
Figure S5 Phylogenetic analysis of MdoMYB121 and abiotic stress-related MYBs from other species. The tree was constructed using the neighbor-joining method of the MEGAS program with 1000 bootstrap replicates.

**Table S1** MYB genes in apple.

**Table S2** Primers for RT-PCR analysis.

**References**

1. Du H, Yang SS, Feng BR, Liu L, Tang YX, et al. (2012) Genome-wide analysis of the MYB transcription factor superfamily in soybean. BMC Plant Biol 12: 106.
2. Jung C, Seo JS, Han SW, Koo YJ, Kim CH, et al. (2007) Overexpression of AtMYB41 enhances stomatal closure to confer abiotic stress tolerance in transgenic Arabidopsis. Plant Physiol 146: 627–635.
3. Duobs C, Stracke R, Grotewold E, Weisshaar B, Martin C, et al. (2010) MYB transcription factors in Arabidopsis. Trends Plant Sci 15: 1360–1369.
4. Krausz HD, Denekamp M, Greco R, Jin H, Levy A, et al. Towards functional characterisation of the members of the R2R3-MYB gene family from Arabidopsis thaliana. Plant J 16: 263–276.
5. Yanhai C, Xiaoyan X, Yuan H, Meihua L, Jingang L, et al. (2006) The transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60: 107–124.
6. Williams O, Nahal H, Voetig RJ, Campbell MM (2009) Expansion and diversification of the Populus R2R3-MYB family of transcription factors. Plant Physiol 149: 981–993.
7. Li Q, Zhang C, Li J, Wang L, Ren Z (2012) Genome-wide identification and characterization of abiotic stress-related MYBs from other species. (DOC)

**Text S1** Semi-quantitative RT-PCR sequencing data that do not have a corresponding EST.

**Text S2 References for MYB genes which have been functionally characterized in Arabidopsis.**

**Author Contributions**

Conceived and designed the experiments: YJH ZHC RKW. Performed the experiments: ZHC RKW ZZJ RJF. Analyzed the data: YJH ZHC ZZJ. Contributed reagents/materials/analysis tools: YJH ZHC ZZJ. Wrote the paper: YJH ZHC.
45. Volkov V, Amtmann A (2006) Thellungiella halophila, a salt-tolerant relative of Arabidopsis thaliana, has specific root ion-channel features supporting K+/Na+ homeostasis under salinity stress. Plant J 48: 342–353.

46. Liu J, Ishitani M, Halffer U, Kim CS, Zhu JK (2000) The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. Proc Natl Acad Sci USA 97: 3730–3734.

47. Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci 14: 460–468.

48. Cominelli E, Galbiati M, Vavasseur A, Conti L, Sala T, et al. (2005) A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. Curr Biol 15: 1196–1200.

49. Lai LB, Nadeu JA, Lucas J, Lee EK, Nakagawa T, et al. (2005) The Arabidopsis R2R3MYB proteins FOUR LIPS and MYB68 restrict divisions late in the stomatal cell lineage. Plant Cell 17: 2754–2767.

50. Dai X, Xu X, Ma Q, Xu W, Wang T, et al. (2007) Overexpression of an R1R2R3 MYB gene, OsMdFT2-2, increases tolerance to freezing, drought, and salt stress in transgenic Arabidopsis. Plant Physiol 148: 1739–1751.

51. Molinari HBC, Marur CJ, Daros E, De Campos MKF, De Carvalho JFRP, et al. (2007) Evaluation of the stress-inducible production of proline in transgenic sugarcane (Saccharum spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiol Plantarum 130: 218–229.

52. Gunes A, Inal A, Alpaslan M, Erslan F, Bagci EG, et al. (2007) Salicylic acid induced changes on some physiological parameters symptomatic for oxidative and mineral nutrition in maize (Zea mays L.) grown under salinity. J Plant Physiol 164: 720–736.

53. Bajji M, Kinet JM, Lutts S (2002) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul 36: 61–70.

54. Abe H, Urano T, Ito T, Seki M, Shinozaki K, et al. (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15: 63–78.

55. Guo YZ, Zhu QH, Chen X, Luo JC (2007) GS12: a gene structure display server. Yi Chuan 29: 1023–1026.

56. Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol 2: 28–36.

57. Li WM, Tao Y, Yao YX, Hao YJ, You CX (2010) Ecopop over-expression of two apple Flowering Locus T homologues, MdFT1 and MdFT2, reduces juvenile phase in Arabidopsis. Biol Plantarum 54: 639–649.

58. Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotechnol 19: 765–768.

59. Kotoda N, Hayashi H, Suzuki M, Igarashi M, Hatsuyama Y, et al. (2010) Molecular characterization of FLOWERING LOCUS T-like genes of apple (Malus × domestica Borkh.). Plant Cell Physiol 51: 561–575.

60. Premachandra G, Saneoka H, Kanaya M, Ogata S (1991) Cell membrane stability and leaf surface wax content as affected by increasing water deficits in maize. J Exp Bot 42: 167–17.

61. Hodges DM, Delong JM, Forney AF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207: 604–611.

62. Bates LS, Waldren RP, Teare ID (1973) Rapid determination of proline for water-stress studies. J Biol Chem 249: 3343–3346.