Genomics 113 (2021) 2605–2613

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Original Article

Sex-specific gene expression in the blood of four primates

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ARTICLE INFO

Keywords:
Primates
Blood
Sex-specific expression
X-Y gene pair
MSY gene

ABSTRACT

Blood is an important non-reproductive tissue, but little is known about the sex-specific gene expressions in the blood. Therefore, we investigated sex-specific gene expression differences in the blood tissues of four primates, rhesus macaques (*Macaca mulatta*), Tibetan macaques (*M. thibetana*), yellow baboons (*Papio cynocephalus*), and humans. We identified seven sex-specific differentially expressed genes (SDEGs) in each non-human primate and 31 SDEGs in humans. The four primates had only one common SDEG, *MAP7D2*. In humans, immune-related SDEGs were identified as up-regulated, but also down-regulated in females. We also found that most of the X-Y gene pairs had similar expression levels between species, except pair *EIF1AY/EIF1AX*. The expression level of X-Y gene pairs of rhesus and Tibetan macaques showed no significant differential expression levels, while humans had six significant XY-biased and three XX-biased X-Y gene pairs. Our observed sex differences in blood should increase understanding of sex differences in primate blood tissue.

1. Introduction

Sex is an important biological trait and results in significant differences between male and female mammals. For example, human females consistently exhibit greater longevity than men and age-related diseases differ between the sexes. Women are more likely to suffer from Alzheimer's disease and osteoporosis, while men are more likely to develop cancer and Parkinson's disease [1,2]. Sex also has widespread effects in many other aspects, such as immune response [3–8], emotion and anxiety [9,10], brain functioning and development [11,12], and lipid and glucose metabolism [13]. Although there are fundamental differences between males and females, the molecular mechanisms responsible for the differences remain poorly understood [14]. The traditional viewpoint attributed the above differences to gonadal sex hormones [15], but recent studies have found that gene expression differences may play an important role, particularly the gene expression of sex chromosomes [14,16,17].

Mammalian sex chromosomes evolved from a pair of autosomes about 300 million years ago [18]. Previous sex chromosome research focused on the X chromosome because it has more genes than the Y chromosome and the escaped X chromosome inactive genes [14,19], with much less focus on the Y chromosome. Since evolving, the Y chromosome decayed rapidly with only 3% of its ancestral genes remaining until stabilizing in the last 25 million years [18]. The Y chromosome was once considered a genetic wasteland, hampered by limited knowledge of the gene content of the ancestral autosomes [18]. Recent studies have suggested that Y chromosome genes became specialized for reproduction since the male-only Y chromosome would have less impact on male-female differences outside the gonads [14,17]. With the improvement of sequencing technology, more extensive studies can be undertaken to determine the role of the Y chromosome in the evolution of male-female differences.

The Y chromosome is structurally divided into three main segments, two pseudoautosomal regions and the male-specific region (MSY). The two pseudoautosomal regions, located on the two ends of the Y chromosome, have identical sequences with X chromosomes. The MSY, also

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https://doi.org/10.1016/j.ygeno.2021.06.007
Received 14 December 2020; Received in revised form 3 May 2021; Accepted 6 June 2021
Available online 8 June 2021

known as the non-recombining region of the Y chromosome (NRY), comprises 95% of the Y chromosome's length. It includes at least 156 transcription units and 78 protein-coding genes that collectively encode 27 distinct proteins or protein families [20]. There are two major types of MSY genes. The first type does not have homologs on the X chromosome, which could result in tissue differences between XX and XY individuals once these MSY genes were expressed. The second MSY gene type has similar but not identical X-linked homologs that escape X chromosome inactivation [14,17,18]. The second type of MYS genes and their X-linked homologs are usually called X-Y gene pairs, which encode proteins that are not identical but similar or at least partially equivalent in function [17,21]. The X-Y gene pairs could encode highly dosage-sensitive regulators of transcription, translation, and protein stability, thus even small sex biases in their gene expressions could result in the male-female gene expression differences across the entire genome [17,18,22,23]. Therefore, investigations of Y chromosome gene expression are vital, particularly these X-Y gene pairs in different tissues, for understanding male-female differences.

More recent studies have indicated that the Y chromosome has retained conserved and dosage-sensitive regulatory genes that are expressed in the testes and in non-reproductive tissues throughout the body [17,18]. This expression in non-reproductive tissues has contributed to the gene expression and phenotypic differences between males and females [14,16,17,24]. In fact, non-reproductive tissue studies on various mammal species have demonstrated that sex affects gene expression differently [1,25–27]. For example, Godfrey et al. [17] analyzed the expression of human Y chromosome genes across 36 non-reproductive tissues to investigate the effect of sex on gene expression. Godfrey et al. [17] found gene expression differences between MSY genes and their X homologs in different tissues, like DDX3Y higher expression in colon, pancreas, and testis than other tissues, while DDX3X only higher express in pancreas than other tissues. The findings of Godfrey et al. [17] highlighted the importance of determining the male-female gene expression differences of MSY genes and their X homologs in non-reproductive tissues. They also used the X-Y-sum gene expression in males and X-X gene expression in females to present the expression levels of X-Y pair genes and showed the difference between males and females [17].

Blood is an important type of non-reproductive tissue, which has been used to study expression patterns [28] and could be used to monitor individuals for different markers [29]. Numerous studies have used blood tissue to investigate, for example, the immune system [30,31], aging [32,33], and diseases [34,35]. In human blood tissues, transcriptome studies found disease-related sex differential gene expressions [36], and even cell-type-specific gene expression [37]. To date, no comparative studies of transcriptome sex-specific gene expression have been performed in blood tissues of multiple primates. Therefore, in this study, we analyzed the blood transcriptomes of 18 Chinese rhesus macaques (*Macaca mulatta*), 18 Tibetan macaques (*M. thibetana*), 65 yellow baboons (*Papio cynocephalus*), and 99 humans (*Homo sapiens*) with the same bioinformatics pipeline. We aimed to explore the male-female gene expression differences in the blood of these four primates to detect the conserved sex-specific differentially expressed genes (SDEGs) across different primates and to investigate the expression profile of Y chromosome genes, especially the gene expression pattern of the X-Y gene pairs.

2. Results

2.1. Data collection and transcriptome assembly

We collected whole peripheral blood samples of 18 rhesus macaques, nine females, and nine males, to systematically investigate transcriptome and expression profile differences between male and female primates. We also downloaded the raw sequencing data of nine female and nine male Tibetan macaques, from our earlier study [33] and downloaded the raw sequencing reads of 63 yellow baboons (37 males and 26 females) and 99 humans (44 males and 55 females) from NCBI (Table S1).

We aligned the clean reads of rhesus macaques and Tibetan macaques to the same reference genome (*M. mulatta,* Mmul_10) separately, with an average mapping rate of 95.61% and 85.95%, respectively. The clean data of yellow baboons were mapped to the baboon genome (Panu_3.0) with 88.65% average alignment rates. The clean data of humans were mapped to the human genome (GRCh38.p13) with 93.83% average alignment rates. Then, all 198 samples were processed with the same bioinformatics pipeline. After removing the low expression genes, these reads were assembled into 18,538 and 20,132 known genes in rhesus and Tibetan macaques (respectively), 35,077 known genes in humans, and 18,277 known genes in yellow baboons.

2.2. SDEGs on autosomes and X chromosome

To gain insights into the gene expression differences between males and females, we identified SDEGs on the autosomes and the X chromosome of all four species with the same bioinformatics pipeline. In total, only seven SDEGs have been identified for each of the three non-human primate species (Table 1), while in humans we identified an abundance of 31 SDEGs (Table S2).

Several of the SDEGs of the three non-human primates were noteworthy. In the rhesus macaque, the gene *AKR1B10* (aldo-keto reductase family 1 member B10) was identified as a female down-regulated SDEG. The protein encoded by *AKR1B10* is overexpressed in liver cancer, smoking-induced adenocarcinoma, and breast cancer in humans [38–40], and has been identified as a biomarker for non-small cell lung cancer [39]. We detected SDEG *HEPH* (hephaestin), which was up-regulated in female Tibetan macaques. The hephaestin protein is a multicopper ferroxidase necessary for iron egress from intestinal enterocytes into the blood circulation and is an important link between copper and iron metabolism in mammals [41,42]. In yellow baboons, the gene *ATP5F1E* (ATP synthase F1 subunit epsilon) was down-regulated in females, which encodes the epsilon subunit of mitochondrial ATP synthase.

For humans, 15 SDEGs were up-regulated in females, while the remaining 16 were down-regulated (Table S2). Six up-regulated SDEGs were located on the X chromosome, and the other nine were on autosomes. There were two up-regulated SDEGs *TSIX* and *XIST*, related to the occurrence of X inactivation, which provides dosage equivalence between males and females [43–45]. In the down-regulated SDEGs, just one SDEG *DGKK* was located on the X chromosome, the remaining 15 were located on autosomes.

2.3. Comparation of SDEGs among the four species

We then performed a comparison of the SDEGs among the four primates (Table S3). The results showed that the four primates had only one common SDEG *MAP7D2* (MAP7 domain containing 2), which was located on the X chromosome and up-regulated in females. The MAP7 domain containing 2 (*MAP7D2*) is one of the members of the MAP7 family and plays vital roles in regulating kinesin-1 that promotes microtubule-based transport of numerous cellular cargoes entry into the axon [46,47]. In addition, there were two SDEGs, *ERCC6L* and ENSMMUG00000049951, shared between two species (*ERCC6L* in Tibetan macaques and humans; ENSMMUG00000049951 in rhesus macaques and Tibetan macaques).

2.4. Expression profiles of the Y chromosome genes

Considering the male-specific trait of the Y chromosome, we analyzed the expression levels of all MSY genes and other protein-coding genes on the Y chromosome in male individuals by using a different pipeline from the X chromosome and autosomes.

Table 1
The SDEGs information of the three non-human primates.

Species	Ensembl ID	Gene name	description	log2FoldChange	padj	up-down (Female)	Chromosome
Rhesus macaque	ENSMMUG00000000192	*MAP7D2*	MAP7 domain containing 2	3.925679506	3.84E-15	up	X
Rhesus macaque	ENSMMUG00000012424	*NTRK1*	neurotrophic receptor tyrosine kinase 1	1.069668334	0.022600888	up	1
Rhesus macaque	ENSMMUG00000021516	*CLU*	clusterin	1.464157028	0.022600888	up	8
Rhesus macaque	ENSMMUG00000049951	ENSMMUG00000049951	None	7.945133637	4.85E-21	up	X
rhesus macaque	ENSMMUG00000064587	ENSMMUG00000064587	None	−3.495941921	0.006093067	down	5
Rhesus macaque	ENSMMUG00000013098	*AKR1B10*	aldo-keto reductase family 1 member B10	−5.014052504	0.008091541	down	3
Rhesus macaque	ENSMMUG00000001713	*CDH20*	cadherin 20	−1.451669644	0.008091541	down	18
Tibetan macaque	ENSMMUG00000049951	ENSMMUG00000049951	None	10.38202754	1.25E-43	up	X
Tibetan macaque	ENSMMUG00000002805	*HEPH*	hephaestin	2.198401394	0.047151412	up	X
Tibetan macaque	ENSMMUG00000000192	*MAP7D2*	MAP7 domain containing 2	2.100598328	0.012123499	up	X
Tibetan macaque	ENSMMUG00000057571	ENSMMUG00000057571	None	1.483185401	0.037553974	up	12
Tibetan macaque	ENSMMUG00000012957	*ERCC6L*	ERCC excision repair 6 like, spindle assembly checkpoint helicase	1.448893688	8.99E-09	up	X
Tibetan macaque	ENSMMUG00000004362	*FCGR1A*	Fc fragment of IgG receptor Ia	1.368791261	0.047151412	up	1
Tibetan macaque	ENSMMUG00000003312	*CHSY3*	chondroitin sulfate synthase 3	−1.52768062	0.037553974	down	6
Yellow baboon	ENSPANG00000003847	ENSPANG00000003847	None	6.609877901	4.42E-49	up	X
Yellow baboon	ENSPANG00000019661	*MAP7D2*	MAP7 domain containing 2	2.143726625	0.009331742	up	X
Yellow baboon	ENSPANG00000007627	*POU5F1*	POU class 5 homeobox 1	1.244472028	0.008121881	up	X
Yellow baboon	ENSPANG00000006634	ENSPANG00000006634	None	−1.093979286	0.013350219	down	10
Yellow baboon	ENSPANG00000023448	ENSPANG00000023448	None	−1.491195143	0.000232437	down	9
Yellow baboon	ENSPANG00000035342	ENSPANG00000035342	None	−3.827304365	1.41E-15	down	20
Yellow baboon	ENSPANG00000011744	*ATP5F1E*	ATP synthase F1 subunit epsilon	−22.59593738	5.42E-11	down	10

In humans, we focused on the MSY genes due to the importance of the male-specific region of the Y chromosome. After referring to past studies [17,18,20,22] and the annotation of the Y chromosome, we obtained 51 MSY genes consisting of 48 protein-coding genes and three MSY genes that were pseudogenes in the human genome (GRCh38.p13) (Table S4). Of these 51 human MSY and protein-coding genes, *RPS4Y1*, *KDM5D*, and *DDX3Y* were the three most expressed genes, whereas more than one half of the rest genes did not express at all in both males and females, like *AMELY*, *VCY*, and so on (Table S5). In rhesus macaques, we obtained 38 protein-coding genes that most of them belonged to MSY genes [18,48] (Table S4). Among them, *RPS4Y1*, ENSMMUG00000055976, and *RPS4Y2* were the top three genes with the most abundant expression levels, and more than ten genes were not expressed at all in all of the male individuals (Table S5).

The reads of Tibetan macaques were mapped to the genome of the rhesus macaque and reads of yellow baboons were mapped to the Y chromosome of the rhesus macaque (see methods). Therefore, we used the same 38 MSY genes for Tibetan macaques and yellow baboons as for rhesus macaques. Same with rhesus macaques, *RPS4Y1*, ENSMMUG00000055976, and *RPS4Y2* were the top three genes with the most abundant expression levels both in Tibetan macaques and yellow baboons (Table S5). The same high expression genes in the four primates in blood tissue showed a concordance with their close relationship.

2.5. Expression levels of X-Y gene pairs

After referring to the expression levels of MSY genes, we found that the MSY genes lacking an X-homolog and belonging to the five multicopy gene families (*BPY2, CDY, DAZ, PRY,* and *XKRY*) were not expressed in the whole blood tissue of humans (Table S5). This is consistent with findings from other tissues, that *BPY2, CDY,* and *PRY* gene families only showed testis-specific expression in humans, and the *XKRY* gene family was not expressed in any tissues [17]. We only found two multicopy gene families in macaques, *CDY* and *DAZ*, according to the human homolog information (Table S4). These two multicopy gene families were not expressed in the whole blood tissue of rhesus and Tibetan macaques (Table S5). Whereas the two MSY genes of the *CDY* family (ENSMMUG00000054832 and ENSMMUG00000050111) were expressed in the whole blood tissue of yellow baboons but with extremely low expression values (Table S5).

Next, due to the vitally important functions of X-Y gene pairs [17,18,23], we investigated the quantitative differences in X- and Y-homolog expression of X-Y gene pairs. In total, we collected 16 and 14 X-Y gene pairs in humans and rhesus macaques, respectively (Table S6) [17,20]. We then analyzed the expression levels of the above X-Y gene pairs in rhesus macaques, Tibetan macaques, and humans. Firstly, we estimated the Y-homolog-to-X-homolog expression ratio (Y/X expression ratio) in each XY sample of the three species (Fig. 1). Through the

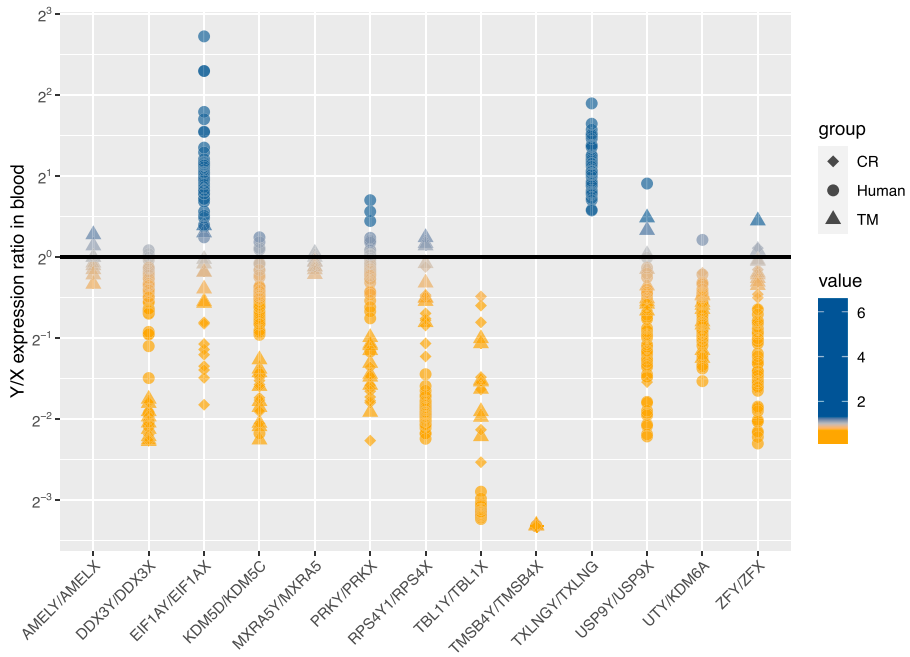

Fig. 1. The Y/X expression ratio level in male individuals of rhesus macaques, Tibetan macaques, and humans. The Y/X expression ratio was estimated as (Y-homolog TPM + 0.5)/(X-homolog TPM + 0.5) in individuals. We deleted the TPM of both Y homolog and X homolog less than one.

Y/X expression ratios, we detected the differences between the three primates. Among these three primates, most of the X-Y gene pairs showed X-biased (Y/X expression ratio < 1) expression levels (Fig. 1). We observed that only two Y homologs (*EIF1AY* and *TXLNGY*) in humans generally expressed higher levels than their X homologs (Y-biased). Overall, the results showed that there were more X-biased expressed genes in the males and the expression pattern in the two macaque species was more similar than in humans (Fig. 1).

Furthermore, we questioned if the observed expression differences within XY individuals of the X-Y gene pairs were also existed between males and females and result in the expression differences between them. Therefore, we performed a statistical analysis of the summed expression level of the Y and X homologs in XY individuals, and only the X homologs in XX individuals in rhesus macaques, Tibetan macaques, and humans (Fig. 2) [17]. As expected, we observed that the X homologs of eight, one, and five X-Y gene pairs showed significant XX-biased (higher in females) expression in humans, rhesus macaques, and Tibetan macaques, respectively, with 1.29-fold to 1.81-fold higher expression levels in females (Table S7). The expression level of the X homologs in the XX individuals was higher than in the XY individuals because the X homologs of these X-Y gene pairs, expressed in most tissues, are not subject to X chromosome inactivation in XX cells [17,19,43].

We then compared the summed expression level of the X and Y homologs in XY individuals and the expression level of the X homolog in XX individuals. We obtained six significant XY-biased (higher in males) X-Y gene pairs (*DDX3X/DDX3Y*, *EIF1AX/EIF1AY*, *KDM5C/KDM5D*, *PRXK/PRKY*, *TXLNG/TXLNGY,* and *USP9X/USP9Y*) in humans (Fig. 2). In particular, the X-Y pair *TXLNG/TXLNGY* had more than a 2-fold XY-biased expression than in XX individuals. We also identified three significant XX-biased (higher in females) X-Y gene pairs (*RPS4X/RPS4Y1*, *KDM6A/UTY*, and *ZFX/ZFY*). Unlike in humans, the expression differences of X-Y gene pairs were not significant in rhesus and Tibetan macaques (Fig. 2).

2.6. RT-qPCR expression data

Finally, we performed reverse transcription-quantitative real-time PCR (RT-qPCR) to verify the gene expression levels of three genes in the rhesus macaque blood transcriptome. The blood samples from the same 18 rhesus macaques were used to validate expression differences between female and male individuals. Three candidate genes (ENSMMUG00000049951, *DDX3Y,* and *RPS4Y1*) from SDEGs and the Y chromosome were randomly selected to be used for RT-qPCR validation (Table S8). As expected, we found significant increases in the expression of *RPS4Y1* and *DDX3Y*, and decreased expression of ENSMMUG00000049951, from female to male (Fig. 3A-C). The expression changes of *RPS4Y1, DDX3Y,* and ENSMMUG00000049951 between the sexes were consistent with the results from blood transcriptomes (Fig. 3D-F).

3. Discussion

3.1. Small gene expression differences between sexes in non-human primate blood tissue

In human blood tissues, transcriptome studies found disease-related sex differential gene expressions [36], and even cell-type-specific gene expression [37]. We also obtained relatively abundant SDEGs in human blood tissue, compared to the non-human primates. However, there were very small sex differences in gene expression in the blood tissue of rhesus macaques, Tibetan macaques, and yellow baboons. Only seven SDEGs were identified in each of the non-human primates, which were located on the autosomes and X chromosome. A possible reason for this human-associated abundance could be the better gene annotation for the human genome, which has assembled 27,932 known genes. This number is 1.38–1.52 fold more than the three non-human primates. Low numbers may be common in non-human mammals since only one gene (*ENSCAFG00000030886*) was significantly associated with sex in the blood tissue of grey wolves [49]. Therefore, it seems understandable that we obtained limited numbers of SDEGs between male and female individuals. We noticed that the detected gene expression differences between males and females could be influenced by factors such as age and sampling time. Since we did not have the information of the sampling time for samples from public database, further studies were needed to investigate its potential effect.

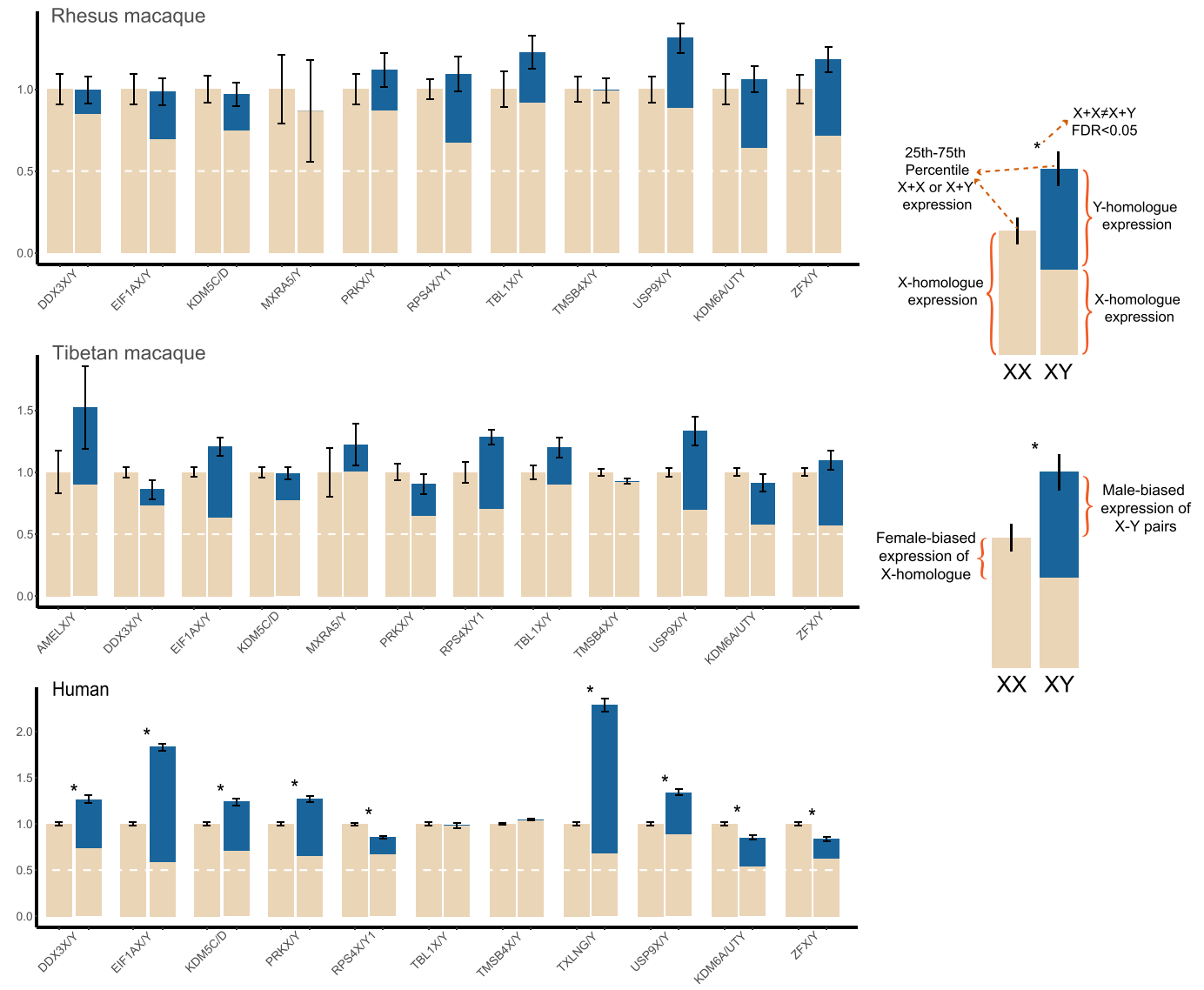

Fig. 2. The expression levels of X-Y gene pairs in rhesus macaque, Tibetan macaques, and humans. We only kept the genes with three or more samples' TPM greater than 0 in male or female individuals. The TPM values of X homologs in females were changed to 1.0, and then the values of X homologs and Y homologs in the males were calculated relative to the values of X homologs in the females. The expression level differences between males and females were assessed with a two-sided Mann-Whitney *U* test to obtain the *P* value, and adjusted by Benjamini–Hochberg procedure.

3.2. SDEGs in human blood tissue

In accordance with our findings, there have been studies that focused on the sex-specific gene expression in blood tissue of healthy humans, and found that females had immune-related sex-specific expressed genes, while some males had renal-cancer-related sex-specific expressed genes [36,50]. However, Jansen et al. [36] identified 582 autosomal genes as sex-specific genes, far more than our 31 SDEGs. Why was there such a big difference in the numbers? Simply, we had different thresholds for screening. Genes with FDR $\leq$ 0.05 and |log2 fold change| $\geq$ 1 were screened as SDEGs in our study (see methods), while Jansen et al. [36] screened only for genes whose *p* values were significant ($p < 1.2e\text{-}6$, Bonferroni corrected at $p < 0.05$, FDR < 6e-5), not for difference multiples (log fold change), and 99% genes had absolute log fold changes smaller than 0.08. We also had over one thousand genes related to sex prior to screening of log2 fold change (Table S9). Nevertheless, several of our SDEGs, such as *XIST*, *TSIX*, *MAP7D2*, *DEFA4*, and *CTSG*, matched the sex-specific genes identified Jansen et al. [36].

In our human SDEGs, the up-regulated (in females) SDEGs *RSAD2* (radical S-adenosyl methionine domain containing 2) encodes interferon-inducible antiviral proteins, which exhibits antiviral activity to viruses including the influenza virus and human cytomegalovirus [51–53]. Numerous studies have shown that sex can affect the immune response to its own antigens and foreign antigens, such as bacteria, viruses, and parasites [3–8]. As early as the last century, studies have shown that females have a greater ability to produce antibodies than men [54,55]. Meanwhile, females are also more likely to develop autoimmune diseases, like Systemic Lupus Erythematosus (SLE) [56,57]. We identified *IFI44L* as an up-regulated SDEG in female blood transcriptomes and the methylation level of the *IFI44L* promoter in blood tissue can serve as a biomarker to diagnose SLE patients [58]. Surprisingly, we identified several immune-related genes down-regulated SDEG in females, such as *DEFA4*, *CTSG*, and *ELANE*, which are involved in fighting off pathogens [59,60]. Despite the greater overall immune response ability in females, there are several immune-related genes expressed higher in males significantly affecting male-female immune system disorder differences.

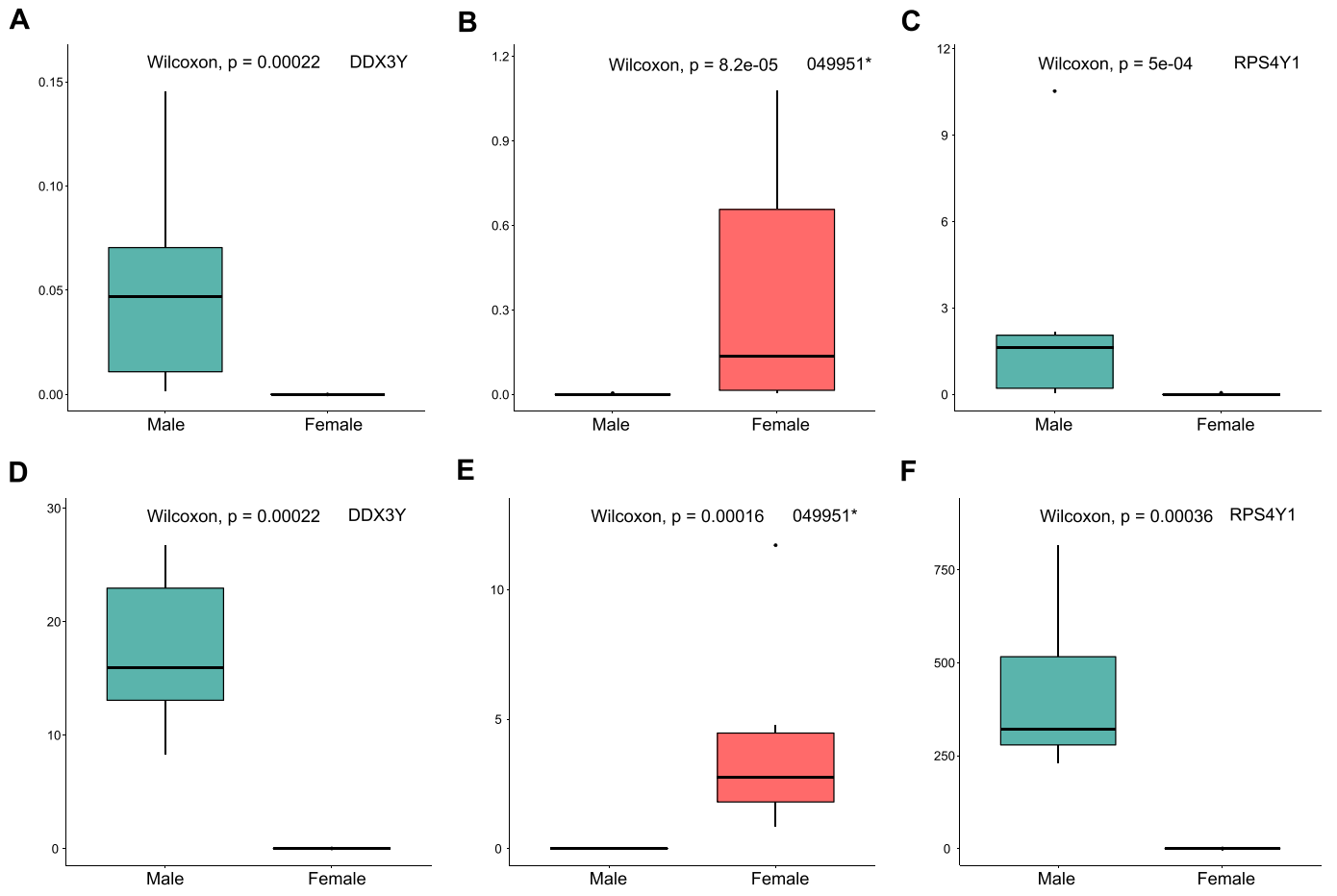

Fig. 3. The result of RT-qPCR and the corresponding transcriptome results of rhesus macaques. A-C. The RT-qPCR result of gene *DDX3Y,* ENSMMUG00000049951, and *RPS4Y1* (from SDEGs or Y chromosome genes) between male and female rhesus macaque groups. D-F. The corresponding transcriptome results. We found significant increases in expression of *RPS4Y1* and *DDX3Y*, but a decreasing expression of ENSMMUG00000049951, from female to male, in the RT-qPCR result and same tendency in transcriptome results.

3.3. X-Y gene pairs expression in primate blood

Most of the X-Y gene pairs showed similar expression levels between species when Y/X expression ratios were analyzed, except pair *EIF1AY/EIF1AX* (Fig. 1). The X-Y pair *EIF1AY/EIF1AX* showed prominent Y-biased in humans but X-biased in the two macaque species (Fig. 1). Godfrey et al. [17] found that *EIF1AY* and *EIF1AX* were expressed at similar levels in most tissues. While Godfrey et al. [17] also detected the elevated expression of *EIF1AY* in the heart, skeletal muscle, pituitary, and spleen. The evolutionary loss of a microRNA target site, miR-1, in *EIF1AY* could result in the tissue-specific upregulation of *EIF1AY* expression in the heart and skeletal muscle [17]. The expression of miR-1 in heart and skeletal muscle regulated the expression of *EIF1AX*. miR-1 is also expressed in blood tissue [61], which might also explain the same Y-biased expression of *EIF1AY/ EIF1AX* in human blood tissue.

In the X-Y gene pairs expression level analysis, we detected six significant XY-biased pairs (*DDX3X/DDX3Y*, *EIF1AX/EIF1AY*, *KDM5C/KDM5D*, *PRXK/PRKY*, *TXLNG/TXLNGY,* and *USP9X/USP9Y*) in human blood tissue. These X-Y pairs showed significant XY-biased expression levels in most other human tissues, especially *EIF1AX/EIF1AY*, *KDM5C/KDM5D*, reported by Godfrey et al. [17]. These X-Y gene pairs showed a consistent expression-biased in multiple tissues. We found that three pairs (*RPS4X/RPS4Y1*, *KDM6A/UTY*, and *ZFX/ZFY*) showed significant XX-bias in human blood tissue (Fig. 2). The pair *RPS4X/RPS4Y1* had the same XX-biased expression levels in other tissues, such as skin, pancreas, and artery [17]. However, the pairs *KDM6A/UTY*, and *ZFX/ZFY* did not have a significant XX-bias in any other tissues [17], except in our blood tissue.

Unexpectedly, the widely expressed gene *TMSB4Y* [17] (in 36 human tissues) was minimally expressed in the blood tissue of humans (Fig. 2). Similarly, *TMSB4Y* was minimally expressed in rhesus and Tibetan macaques. These differences between blood and other tissues indicate that there are tissue-specific expression differences. We did not detect any significant X-Y gene pair expression differences between males and females in the two macaques (Fig. 2). However, Godfrey et al. [17] found that a large proportion of human X-Y gene pairs had non-significant gene expression differences in many tissues. So far, X-Y gene pair expression studies in macaque non-reproductive tissues are limited, and therefore we had no direct comparison. Thus, the observed differences between macaques and humans could be species/genera-specific, but further studies will be necessary for verification.

In conclusion, we identified the seven and 31 SDEGs, respectively in three non-human primates and humans, and had a deep insight into the X-Y gene pairs expression in the four primates. The four primates had only one common SDEG, *MAP7D2*. In humans, we identified immune-related SDEGs both in up-regulated and down-regulated in females, even though females typically have greater immune response capacity than males. We found that most of the X-Y gene pairs had similar expression levels between species, except pair *EIF1AY/EIF1AX*. The humans had six significant XY-biased and three XX-biased X-Y gene pairs, but rhesus and Tibetan macaques had no significant differential expression X-Y gene pairs. Our observed sex differences in blood should increase understanding of sex differences in primate blood tissue.

4. Materials and methods

4.1. Sample collection

We collected whole peripheral blood samples during a routine examination of 18 healthy captive Chinese rhesus macaques at the Sichuan Green-house Biotech Co., Ltd. in Meishan, Sichuan, China, to systematically compare transcriptomes and expression profiles between male and female primates. All rhesus macaques were housed in the same captive condition. This study was approved by the Ethics Committee of College of Life Sciences, Sichuan University (No. 20200529001), and the sample collection and utility protocols were carried out in strict adherence to the guidelines of the management committee of experimental animals of Sichuan Province, China (SYXK-Sichuan, 2019–192). We preserved the fresh blood samples in specialized blood collection tubes (PAXgene Blood RNA tubes) for four hours at room temperature (about 18–20 °C) and then transferred the samples to −20 °C for 24 h. After that, we stored all the samples at −80 °C until RNA extraction.

Tibetan macaques, yellow baboons, and humans were not directly sampled for this study, with raw sequencing data sourced from previous studies. Sequencing, health status, and other information were sourced about these three primates to avoid possible biases generated from different procedures or samples. The raw blood transcriptome sequencing data from 18 Tibetan macaques (nine females and nine males) were collected from our earlier study [33] (NCBI accession number SRP181993). The whole blood samples of Tibetan macaques were collected with the same protocol and same PAXgene Blood RNA tubes as rhesus macaques [33]. The samples were sequenced on Illumina HiSeq 2000 with 150 bp paired-end reads. The blood transcriptome raw sequencing data of 37 male and 26 female yellow baboons were obtained from NCBI [62] (accession number SRP050442). These whole blood samples were collected with a very similar pipeline and tubes (PAXgene Vacutainer tubes) as our studies [62]. The samples were sequenced on Illumina Genome Analyzer II with 76 bp single-end reads. Finally, the raw sequencing data of 99 healthy humans (44 males and 55 females) were downloaded from NCBI [63] (accession number SRP214077). Aguirre-Gamboa et al. [63] collected the whole blood samples and then extracted the total RNA to perform the RNA-seq on Illumina HiSeq 2000/2500 with 100 bp paired-end reads. The detailed information of all the samples used in this study is shown in Table S1.

4.2. Library preparation and RNA sequencing

Sequencing of rhesus macaque samples involved using a total of 3 μg RNA per sample as input material for the RNA sample preparations. Extracting total RNA followed the manufacturer's PAXgene Blood RNA kit manual and assessing RNA quality used an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was processed with the GlobinZero kit (Epicentre, Illumina, Madison, WI) and purified with a modified Qiagen RNeasy MinElute (Qiagen Inc., Valencia, CA) cleanup procedure or ethanol precipitation. Samples with RIN (RNA integrity number) values higher than 7.5 were used for library construction, sequencing, and real-time quantitative PCR (RT-qPCR). Library preparation and all sequencing runs were performed following the manufacturer's instructions. We used a strand-specific kit from Epicentre (ScriptSeq v2 Library Prep kit, Illumina, Madison, WI) with ScriptSeq Index PCR primers (Epicentre, Illumina, Madison, WI) to transform the single-stranded RNA into cDNA in a reverse transcript PCR approach. In short, each cDNA sample was sonicated to fragments of 300–500 bp in size and applied to paired-end libraries generation, then we used the Illumina NovaSeq 6000 to sequence all of the libraries with a paired-end sequencing length of 150 bp (PE150) at Novogene (Beijing, China).

4.3. Read alignment and quality control

High-quality reads (clean reads) were obtained by using NGS QC Toolkit v2.3.3 [64] for each sample with stringent criteria (high-quality paired reads with more than 90% of bases with Q-value ≥30 were retained) to remove the low-quality paired-end reads or reads containing adaptors. Processed reads from rhesus macaques and Tibetan macaques were mapped to the same reference genome (*M. mulatta,* Mmul_10) using HISAT2 v2.1.0 [65]. In the same way, the processed reads of humans were mapped to the human genome (*Homo sapiens*, GRCh38.p13) and the processed reads of yellow baboons were mapped to the baboon genome (*Olive baboon,* Panu_3.0, without Y chromosome) with rhesus macaque Y chromosome information from Mmul_10. Each of the alignment output files was assembled into separate transcriptomes using StringTie v1.3.6 [66], producing a transcript GTF file. For each species, three specific Ensembl GTF files (GRCh38.99 with 60,617 annotated genes for humans, Mmul_10.98 with 34,847 annotated genes for rhesus macaques and Tibetan macaques, Panu_3.0.99 with 28,276 annotated genes for yellow baboons) were used separately as the reference annotation file to guide the assembly process to obtain the expression value of TPM (transcripts per million) and raw read counts for each gene and transcripts. The genome sequences and annotations were downloaded from Ensembl release-98 (www.ensembl.org).

4.4. Identification of SDEGs on autosomes and X chromosome

We did not analyze the SDEGs from Y chromosome SDEGs because only males have a Y chromosome. Whereas we used the DESeq2 R package [67] to perform the differential expression analysis on autosomes and the X chromosome, which took the raw read counts as the input. To identify the SDEGs, we divided the samples in each species into two groups based on sex. However, individuals in each group have different ages, which could also affect the gene expressions [33,68]. Therefore, we used sex as the primary factor and age as the batch factor to form the DEseq Data Set design formula to control for age. All statistical test results were corrected for multiple testing with the Benjamini-Hochberg false discovery rate (FDR ≤ 0.05) and an absolute value of log2 fold change ≥1 was used to determine the significant differences in gene expression.

4.5. Gene expression of Y chromosome genes

We extracted the genes on the Y chromosome and performed additional calculations. The TPM expression value was used to represent the level of gene expression after normalization. For these genes, the difference in the male and female expression levels was assessed with a two-sided Wilcoxon signed-rank test (Python function: scipy.stats.wilcoxon). After obtaining *P*-values for all the genes, these P-values were adjusted for multiple hypotheses by using the Benjamini–Hochberg procedure (Python function: statsmodels.stats.multitest.multipletests, method = "fdr_bh").

4.6. Expression levels of X-Y gene pairs

Next, we investigated the expression of MSY genes with X homologs, especially the X-Y gene pairs that were normally expressed in the same tissues as their corresponding X homologs. We only retained the genes that expressed in more than one-third (TPM greater than 0) in males or females, and then the difference in the male and female expression levels was assessed with a two-sided Mann-Whitney *U* test (Python function: scipy.stats. mannwhitneyu). After obtaining P-values for all genes, these P-values were adjusted for multiple hypotheses using the Benjamini–Hochberg procedure (Python function: statsmodels.stats.multitest. multipletests, method = "fdr_bh"). To characterize the quantitative differences in X- and Y-homolog expression of these X-Y gene pairs, we applied the method described by Godfrey et al. (2020) [17]. For a given X-Y gene pair, the Y/X expression ratio was estimated as (Y-homolog TPM + 0.5)/(X-homolog TPM + 0.5) in individuals. When we analyzed the X-Y gene pairs expression in females and males, we set the TPM

value of X homologs in females as 1.0. We then calculated the X and Y homolog values in the males relative to the values of their X homologs in the females [17].

4.7. Real-time quantitative PCR (RT-qPCR)

The blood samples of the same 18 rhesus macaques that were used for RNA-seq were also examined for the expression level of several mRNAs using a real-time PCR assay developed in-house [69]. The isolated RNA was converted to double-stranded cDNA, using iScript cDNA Synthesis Kit (Biorad, USA). After the cDNA synthesis, we used real-time PCR performed on a 7500 real-Time PCR System (Thermo fisher, USA) to quantify the expression levels of the three mRNAs. All primer sequences are shown in Table S10.

A total of 20 μl reaction mix for the RT-qPCR was comprised of 10 μl FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, USA), 5 μl cDNA served as a template and 4 μl ddH2O, 0.5 μl forward primer (10 pmol/μl), and 0.5 μl reverse primer (10 pmol/μl). In each run, negative controls containing water as a template were also included. The cycling conditions were as follows: 1 cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s; and a final cycle of 95 °C for 15 s, 60 °C for 15 s, and then gradually increased to 95 °C in 30 min at a ramp rate of 2%. The expression levels of the mRNAs were then analyzed using the relative quantification (delta-Ct) method [70]. The housekeeping gene, *GAPDH*, was included as an internal control in all RT-qPCR runs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.06.007.

Author statement

Jiao Wang, Yue Lan, Lewei He and Ruixiang Tang performed the bioinformatics analyses and collected the samples; Yuhui Li, Yuan Huang, Shan Liang and Zhan Gao performed the experiments; Jiao Wang and Yue Lan wrote the manuscript; Bisong Yue, Miao He, Tao Guo and Zhenxin Fan revised the manuscript; Zhenxin Fan and Tao Guo designed and supervised the study.

Availability of data

The raw sequencing reads from this study have been submitted to the NCBI with the project accession PRJNA628554.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the Sichuan Science and Technology Program (2020YJ0303) and the National Natural Science Foundation of China (32070413). We thank Mr. Guanglun Lei at Sichuan Green-house Biotech Co., Ltd. for sample collection.

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