



# Article MdPP2C24/37, Protein Phosphatase Type 2Cs from Apple, Interact with MdPYL2/12 to Negatively Regulate ABA Signaling in Transgenic Arabidopsis

Ying-Ying Liu<sup>†</sup>, Wen-Sen Shi<sup>†</sup>, Yu Liu<sup>®</sup>, Xue-Meng Gao, Bo Hu, Hao-Ran Sun, Xiao-Yi Li<sup>®</sup>, Yi Yang<sup>®</sup>, Xu-Feng Li, Zhi-Bin Liu and Jian-Mei Wang \*

Key Laboratory of Bio-Resources and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610065, China

\* Correspondence: wangjianmei@scu.edu.cn

+ These authors contributed equally to this work.

Abstract: The phytohormone abscisic acid (ABA) plays an important role in the ability of plants to cope with drought stress. As core members of the ABA signaling pathway, protein phosphatase type 2Cs (PP2Cs) have been reported in many species. However, the functions of MdPP2Cs in apple (*Malus domestica*) are unclear. In this study, we identified two PP2C-encoding genes, *MdPP2C24/37*, with conserved PP2C catalytic domains, using sequence alignment. The nucleus-located *MdPP2C24/37* genes were induced by ABA or mannitol in apple. Genetic analysis revealed that overexpression of *MdPP2C24/37* in *Arabidopsis thaliana* led to plant insensitivity to ABA or mannitol treatment, in terms of inhibiting seed germination and overall seedling establishment. The expression of stress marker genes was upregulated in *MdPP2C24/37* transgenic lines. At the same time, *MdPP2C24/37* transgenic lines displayed inhibited ABA-mediated stomatal closure, which led to higher water loss rates. Moreover, when exposed to drought stress, chlorophyll levels decreased and MDA and H<sub>2</sub>O<sub>2</sub> levels accumulated in the *MdPP2C24/37* transgenic lines. Further, MdPP2C24/37 interacted with MdPYL2/12 in vitro and vivo. The results indicate that *MdPP2C24/37* act as negative regulators in response to ABA-mediated drought resistance.

Keywords: Malus domestica; abscisic acid; drought stress; MdPP2C; MdPYL

# 1. Introduction

The apple tree (*Malus domestica*) is one of the most important fruit trees in the world, and its yield is greatly affected by adverse environmental cues, such as drought stress [1,2]. As the world's largest apple producer, China accounts for 50% of the world's apple production, among which '*huashuo*' is an elite hybrid cultivar [3]. Whole genome sequencing analysis has revealed accurate apple gene sequences and provides an effective method to study the function of stress-responsive genes [4,5]. Thus, the study of the apple's resistance to drought stress is urgently needed to cultivate various apple trees with excellent traits, such as '*huashuo*'.

Unlike animals that can escape adverse environments, sessile plants have developed a series of defensive mechanisms to deal with various abiotic stresses, such as drought, cold, and heat [6]. As a major environmental factor, drought stress affects the geographical distribution of agricultural productivity and environmental sustainability, causing abscisic acid (ABA) accumulation in plants. Further, the phytohormone ABA regulates many physiological processes, including plant development, seed maturation, stomata movement, and signal transduction under abiotic stress [7,8].

Many key components in the ABA signaling pathway have been identified at the molecular level, including ABA receptors, group A type 2C protein phosphatases (PP2Cs), SnRKs (sucrose non-fermenting-1 related protein kinases), transcription factors, and ion



Citation: Liu, Y.-Y.; Shi, W.-S.; Liu, Y.; Gao, X.-M.; Hu, B.; Sun, H.-R.; Li, X.-Y.; Yang, Y.; Li, X.-F.; Liu, Z.-B.; et al. MdPP2C24/37, Protein Phosphatase Type 2Cs from Apple, Interact with MdPYL2/12 to Negatively Regulate ABA Signaling in Transgenic *Arabidopsis. Int. J. Mol. Sci.* 2022, 23, 14375. https://doi.org/ 10.3390/ijms232214375

Academic Editor: Shaoliang Chen

Received: 9 October 2022 Accepted: 16 November 2022 Published: 19 November 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). channels [9–11]. When ABA receptors PYR1/PYL/RCAR (pyrabactin resistance 1/PYR1like/regulatory components of ABA receptor) perceive ABA, they bind directly to PP2Cs. This reaction inhibits the activity of PP2Cs. Later, the inhibited PP2Cs release the bound SnRKs, causing SnRKs to shuttle from inactivated status to activated status in order to phosphorylate the downstream transcription factors ABRE (ABA-responsive element binding) proteins or AREB/ABF (ABRE-binding factors) [11–17]. The phosphorylated transcription factors activate or inhibit ABA-responsive genes through the DNA-binding element on their promoter regions [18,19].

PP2Cs, which belong to the largest protein phosphatase family in plants, are the core components of ABA signaling. The following PP2Cs have been identified: ABI1 (ABA-INSENSITIVE 1), ABI2, HAB1 (ABA-hypersensitive 1), HAB2, AHG1 (ABA HYPERSENSITIVE GERMINATION 1), and PP2CA/AHG3 [20,21]. A recent study showed that AtPP2CF1 (PP2C ISOFORM), an ABI1-interacting protein in *Arabidopsis*, is insensitive to ABA during seed germination and under drought stress [22]. AtAHG1, which encodes a protein phosphatase 2C in mutant *ahg1*, is more hypersensitive to ABA, salinity, mannitol, glucose, and sucrose at the seed germination and post-development stages than its analogue AtAHG3 in mutant *ahg3* [23]. In maize, *ZmPP2C-As* are dramatically induced by multiple stresses. *ZmPP2C-A2* and *ZmPP2C-A6* overexpression causes hyposensitivity to exogenous ABA and NaCl treatments at the germination stage and negatively regulates the drought stress response when exposed to drought stress [24]. In wheat, *TaPP2C-a10* interacts with the dormancy genes *TaDOG1L1* (DELAY OF GERMINATION 1) and *TaDOG1L4* (DELAY OF GERMINATION 4) and decreases tolerance to drought stress in transgenic *Arabidopsis* [25]. Although 128 MdPP2Cs have been identified [26], their functions are still unclear.

In this study, MdPP2C24/37, two group A PP2C genes in apple (huashuo), were cloned and their functions in ABA signaling were identified. Under drought stress, signal transduction consists of the osmotic homeostasis signaling pathway [27,28]. Therefore, this research studied osmotic stress induced by mannitol. RT-qPCR analysis revealed that MdPP2C/37 were induced by ABA or mannitol. Compared with WT plants, MdPP2C24/37 overexpression lines in Arabidopsis were hyposensitive to ABA or mannitol at the seed germination and seedling establishment stages. Transcriptional analysis showed that stress-mediated genes, represented by DREB2A and KIN2, were upregulated in the MdPP2C24/37 overexpression lines under control conditions. Meanwhile, ABF4 and RD29A were only upregulated after plants were treated with exogenous ABA or mannitol. When exposed to drought stress, all of the transgenic plants exhibited inhibited stomatal closure, significantly higher water loss rates, and lower survival rates than WT plants. Under drought stress, MDA and H<sub>2</sub>O<sub>2</sub> levels accumulated while chlorophyll levels decreased more rapidly in the overexpression plants than in WT plants. Furthermore, MdPP2C24/37 interacted with MdPYL2/12 in vitro and vivo, respectively. Taken together, these results indicate that MdPP2C24/37 act as negative regulators in ABA signaling and are involved in ABA-mediated drought resistance.

#### 2. Results

# 2.1. Structure and Homology Characterization of MdPP2C24/37

In apple, *MdPP2C24* (*LOC103407751*, *MDP0000883465*) encodes a 408-amino acid protein on the third chromosome, while *MdPP2C37* (*LOC114826111*, *MDP0000203818*) encodes a 424-amino acid protein on the seventh chromosome. In this study, based on the NCBI website, both MdPP2C24/37 were predicted to possess conserved PP2C catalytic domains (Figure 1A). Using MEGA 7, a phylogenetic tree was generated according to the highly conserved PP2C homologous protein sequences (Figure 1B, Supplementary Figure S1). In addition, the phylogenetic tree analysis revealed that MdPP2C24 shared 56.69% identity with AtPP2C3, and MdPP2C37 shared 60.55% identity with AtPP2CA (Figure 1B). These results indicated that *MdPP2C24/37* belong to the conserved PP2C gene family in plants.

3 of 16



# PP2C catalytic domain

В

M. domestica\_XP\_028961732 1 / PP2C 37 M. domestica\_XP\_028956535 1/MdPP2C24 A. thaliana\_NP\_1870491 //PP2C 3 A. thaliana\_NP\_1870481 //PP2C 3 A. thaliana\_NP\_1870481 //PP2C 3 G. max\_XP\_003550788 //PP2C 37 E. salsugineum\_XP\_006406984 //PP2C 24 P. persica\_XP\_007218057 //PP2C 37 B. napus\_XP\_013684505 //PP2C 37 P. avium\_XP\_016190182 //PP2C 37 P. avium\_XP\_0218137801 //PP2C 37 P. avium\_XP\_0218137801 //PP2C 37 C. arietinum\_XP\_02191726 //PP2C 37 C. arietinum\_XP\_02191726 //PP2C 37 P. dubics\_XP\_02419671 //PP2C 37 P. dubics\_XP\_024196171 //PP2C 37

M. domestica XP\_028961732.1 /PP2C 37 M. domestica XP\_028965535.1/MdPP2C24 A. thaliana\_NP\_180499.1/PP2C3 A. thaliana\_NP\_187748.1/PP2C3 A. lyrata subsp. lyrata\_XP\_002872013.1 PP2C 24 G. max\_XP\_003552783.1/PP2C 37 E. salsugireum XP\_006403864.1/PP2C 24 P. parsica\_XP\_007218057.1/PP2C 37 B. rapus\_XP\_013664505.1/PP2C 37 P. avium\_XP\_016190182.1/PP2C 37 P. avium\_XP\_021813780.1/PP2C 37 P. avium\_XP\_021813780.1/PP2C 37 P. avium\_XP\_021813780.1/PP2C 37 C. arketinum\_XP\_02191726.1/PP2C 37 P. dubcis\_XP\_034204911.1/PP2C 37 P. dubcis\_XP\_034219617.1/PP2C 24

M. domestica\_XP\_028961732.1 /PP2C 37 M. domestica\_XP\_02895535.1/MdPP2C24 A. thaliana\_NP\_187148.1 /PP2C3 A. thaliana\_NP\_187148.1 /PP2C3 G. max\_XP\_003562788.1 /PP2C37 E. salsugineum\_XP\_00640984.1 /PP2C 24 P. persica\_XP\_007218057.1 /PP2C 37 B. napus\_XP\_007218057.1 /PP2C37 B. napus\_XP\_013664505.1 /PP2C37 P. avium\_XP\_00181370.1 /PP2C37 P. avium\_XP\_013664505.1 /PP2C24 R. chinensis\_XP\_02181570.1 /PP2C24 R. chinensis\_XP\_02181572.1 /PP2C37 C. arietinum\_XP\_0219521.1 /PP2C37 C. arietinum\_XP\_02191726.1 /PP2C37 C. arietinum\_XP\_02191726.1 /PP2C37 C. arietinum\_XP\_02191726.1 /PP2C37 P. dubis\_XP\_034203491.1 /PP2C37 P. dubis\_XP\_034219617.1 /PP2C37 P. dubis\_XP\_034219617.1 /PP2C37

M. domestica, XP\_028961732.1 /PP2C.37 M. domestica, XP\_028956555.1 MidPp2C24 A. thaliana, NP\_18098.1 /PP2C3 A. thaliana, NP\_18098.1 /PP2C3 A. thaliana, NP\_18098.1 /PP2C37 E. saksugineum, XP\_002605921.1 PP2C24 P. persica, XP\_00720539.3 / IPP2C24 P. persica, XP\_00720539.3 / IPP2C24 P. persica, XP\_00720539.1 /IPP2C37 P. avium, XP\_0018076.1 /IPP2C37 P. avium, XP\_001813780.1 /IPP2C37 P. avium, XP\_001813780.1 /IPP2C24 R. chinensis, XP\_004199821.1 /IPP2C24 R. chinensis, XP\_0042199821.1 /IPP2C24 R. chinensis, XP\_0042199821.1 /IPP2C237 C. arietinum, XP\_004219671.1 /IPP2C37 C. arietinum, XP\_004219671.1 /IPP2C37 P. dukicis, XP\_00420491.1 /IPP2C37 P. dukicis, XP\_004219617.1 /IPP2C37

NACHO COVICE SEASATTTTTTTA DASSETSE		MANCE TAENCERER	I VESI SSVECNAAESS	KEGI EESKESETVRVVEDDAGVK	102
	REAL FLAR EKEVSG	VAPPAPESPECSENCKK	AASETASI PSRELESALI	CSCSSDE EKI PPS	89
MAEL CYEVUT DACES SVYESTEALSE	REPRE CTVNEEDWEK	NCKRSK	CEALATRYSSI PRSSRED	ESDC	69
ACI CCVVCETEPA APVOSTSRASL	RERUDUL PSI KI VAD.			VRSENKKAR	83
AD OYEVVTDASPS SVYCSTPPHSR	RRCRF CTVNSEDWEK	NCKRSK	CEGLATROSSI SRRSCDD.	FSGSRLLKT	74
NACI CCOVVCECCTP APLEFTPRPSR	RESLDI LP. LKYI ADNP		LNLRDCDNAI ENCDESTG	FKVTKKESKVDYDDDVVS	90
NADI OF EVVT DASSS SAFESRPLNSV	RECREPND. CTVVCEDWKK	NOKRNK.	CEDLAKTCSLNSPSSKED		77
MAEL CCOVVSESETSS NPCECSSRAAR	RR <mark>RN</mark> EI RR. YKFVSG	VASP PENAEKRKK.	VEACTTSV. SRDLDNALE	CSGSSDE. EKVVAS	84
NAGI CCGVVGEAEAS TTI DASPRNSR	RR <mark>RL</mark> EL <mark>L</mark> P. L <mark>K</mark> YI AD	. VAVKPS TVENGRKRPK.	I DLYCSSSSVPCKAVESSSE	VKSKSESEGSKLNATVRFGDDDFDAV	102
ADI CYEVVTDAS AYESRPSHS	RECREPNDETVVNCEEVER	KNFKRNK.	LEALTVRNENVSGESP		65
MAGI CCGVVCEGEAT API EPSCRPSR	RES <mark>L</mark> DLLS. LELATOVA	. TVAAP EASRKRCR.	MDL CAVPRERGNVAENRGS.	FVAGCNKEDGSKCLVNNEDGTVS	97
NAGI CCGVVCEAEAS TTI EASSRNSR	RR <mark>RL</mark> EL <mark>L</mark> P. L <mark>K</mark> YI AD	. NAVK <mark>P</mark> S TV <mark>EN</mark> SRKRCK.	I DLYRSSSVPHNAVESSSE	AKSKSESEGSKLNATVRFGDDDFDAV	102
NTEL CCGVVSESETSS NPCECSSRAAR	RR <mark>RN</mark> EI RR. Y <mark>K</mark> FVSG	VAS <mark>P</mark> P <mark>EN</mark> AEKRKK.	VEACFTSV. SRDLDNALE	FSGSSDE. EKVVAS	84
MAEL CYGVL CENE. AS SACETSARAAR	RR <mark>RN</mark> EI RR. F <mark>K</mark> FVSG	VAAP. AEST VNAEKRKR.	LECCTVSRGNEKCGDEEK	GNLVKI ESCEGVSS	88
MAGI CCGLVCESETS API EPSSETS	RR <mark>RL</mark> DL <mark>L</mark> S. I <mark>K</mark> CI AADH	. MALCC AVDNGRKRCK.	LDLLRSLS RDCEKR	EVS. DATMRLDGEEAVI S	88
MAGNOCGVVCETDSP A SSRSSR	RENLDLLP. FKYI ADNA	VSCP ST SRKRRCF	PDLCTPKDFESCEETSGDCGKDKN	NKKKKDESKVN DKVI S	90
NACI CCOVVCEAEAS TTI DASPRNSR	RE <mark>RL</mark> EL <mark>L</mark> P. L <mark>K</mark> YI AD	. VAVK <mark>P</mark> S TV <mark>EN</mark> GRKRCK.	I DLYRSSSLVPCKAVESSSE	VKSKSESEGSKLNATVRFGDDDFDAV	102
MAEL CCOVVSESETSS NPCECSSRAAR	RRRNEI RR. YKFVSG	VAS <mark>P</mark> P <mark>EN</mark> AE <mark>KR</mark> KK.	VEACTTSV. SRDLDNALE	CSGSSDE. EKVVAS	84
m c	rr	k			
				_	
DARGDDVSE. SVRVENEAVCA LVCES	PRFGNTSVCGRRRDMEDAV	SI HESELCKENPESNETHE	CONFIDENCESH VAL KOKDRUYET VKO	ELETEGES VOVKDANERSFLKM	213
R KKSNKFRASTSSNVTE ASREN	LRYCVSSVCCRRRDMEDAV	SVHESECREI RDSAACLEY	GVYDGFGCSFVATKOREWNHELVKD	EVES NEEVETANERSEVEM	194
	PRYCVSSVCCRRREMEDAV	AI HESESSEK, NSEFECT	GVYDGHGCSHVAARCRERLHKLVCE	ELSSDNEDEE EVETTNERSETEM	161
S AVTNSNSVTEAES FFSDV	PKI OTTSVCGRRRDMEDAV	SI HESELCEN SENHEE	CONFIDERCENTRERCERLED VKR	EVEVN. AS DEVTETNVKSFCKM	184
ETVRGNVEV <mark>S</mark> GES	PRYGVSSVCCRRRENEDAV	AI HESESSRK, NSEYPOLY	GVYDGHGCSHVAARGRERLHKLWCE	ELSSDGEEEE EVKTTNERSFTRM	173
E TKNVTVS EVEE ES	EKFOVTSVCGRRRDNEDSV	SVRECETCEF	CVFDCFCCSFVAT MCKERLHEI MNE	ELESAREN LEWKLTMENGEARM	183
K ERVREDDVSGESPATE VS	KRYGVSSVCGRRREMEDAV	AL HESESSEK, NSEYSOLY	CVYDCHCCSHVAARCRERLHKLVCE	ELNSDEEEVE EWKTTNERSETRM	182
L KI AAKCRPSTSSNI PE AFSEF	PKYGLASVCGRRRDMEDAV	SI HESECRESETDEKLEY	GVYDCFCCSFVATRCRERLFELVRC	EVES NEEVETANERSEVEM	189
N GDHCESKSVRVDNEAVCVC ALCES	PREGNTSVCGRRRDMEDAV	SI HESEYCNDGEDSNGALEY	ICVFDGHGCSHVALKCKDRLHEI VKC	ELETEGAFI OVKCAMERSFAKM	213
VTEAS	PRYGVSSVCGRRREMEDAV	AL HESESS ESEYPORT	GVYDGFGCSFVAARGRERLFKLVCE	ELHSDREEEEH EVRKTMERSFTRM	155
E TKRSKVSSCLSLTAEGLGNEKVFEED	FREGUT SVCGRRRD MEDSV	CVRPSFCDES FRGFFY		EI SCAKKDEC. ELEWKSVNEHOFLRM	210
NTRODHCESKSVRVDNEAVCVC ALCOS	PREGNTSVCGRRRDMEDAV	SI HESEYCNDGPDSNGAFEY	GVFDGFGCSFVALKCKDRLHEI VKC	ELETEGGY I OWKGANERSFAKM	215
L KI SAKCRPSTSSNI PE ASSEL	PKYGLASVCGRRRDMEDAV	SI HESECRENRET VOKLEY	GVYDGHGCSHVATROKERLHELVRD	EVES MEEVETANKRSEVEM	189
LTI SSKCPFTSAPVLNPD	AKEGNVSVCGRRRDMEDAV	SI HESECREDCETANNLEY	AVYDGEGCSEVATRORERFFEVVKE	ELENK A LIFEVCSALERSITEM	191
R GESKAL FAENEVVVPPR VVREP	PREGCISVCGRRRDMEDAV	SI HETL CRNDASDSDGCHE	IGVYDGHGCSHVALKOKDRUHEI VKE	DLERC. SV VOVKETNERSFSKM	196
E NKPSTEG SLEVG EF	PKFGVTSVCGRRRDMEDSV	SVKESESCEP FEY	GVEDGEGCSEVATNCKERLEELVKE	EI NEACEN LEWKNTNCCCFARM	185
N GDFCES. SVRVENEAVCVC ALCES	-REGATSVCGRRRDMEDAV	SI HESEYCNDGEDSNGALE	CONFIDERCESEVAL KOKORL HEI VKO	ELETEGAFI OVKGANERSFARM	212
L KI AAKCRPSTSSNI PE AFSEL	EKYCLASVCCRRRDMEDAV	SI HESECRESET DOKLEY	GVMDICHCCSHVATRORERLHELMRI	EVES	189
	g svcgrrr med v	p h	vdghgcshva c v	w fm	
	g svcgrrr med v		v dghgcshva c v		207
DEEVC. EGNLKACSSN	g svcgrrr med v CDAVCSTAVVAVVTPEKIV SEAACSTAVVAVV ABPRIV	P h	v dghgcshva c v S <mark>S</mark> DHKPDRPDELVRI EAAGGRVI YV SVDHKPDRPDELNRI CEAGGRVI YV	W T m	327
DEEV.C. EGNLKACSSNGRGELGTE DKEVVAVGREGGI GVSNGRGELGTE DKEVVAVGREGGI GVSNGRGELGTE	g svcgrrr med v CDAVGSTAVVAVVTPEKI V SEAAGSTAVVAVI APDKI V CDSVGSTAVVAVI APDKI V	P h VSNCGDSRAVLCRSGAAV VANCGDSRAVLCRNGKAFF VANCGDSRAVLCRNGKAFF	V dghgoshva c V SSDHKFDRPDELVELEAAGGEVI VV SVDHKPDRPDELNELGAGGEVI VV	w t m D <mark>CA</mark> RVL CVLANSRAI CDNYLKFYVI S DCFRVL CVLANSRAI CDNYLKFYVSC DCFRVL CVLANSRAI CDNYLKFYVSC	327 309 275
DEEV.C. EGNLKACSSN	g svogrrr ned v CDAVCSTAVCAV TPERI V SEAACSTAVCAVI APDRI V CDSVCSTAVCAVI TPERI CDSVCSTAVCSVI TPERI	P h VSNCGDSRAVLCRSGAAVR VANCGDSRAVLCRNGKAFP VANCGDSRAVLCRNGKPVR VSNCGDSRAVLCRNGKPVR	V dghgcshva c V SSDHKPDRPDELVR EAAGGRVI YW SVDHKPDRPDELNR CEAGGRVI YW STDHKPDRPDELDR EGAGGRVI YW STDHKPDRPDELUR CGAGGRVI YW	W T m DCPRUL QULANSRAI (CDNYL KPYVI S DCPRUL QULANSRAI (CDNYL KPYVSC DCPRUL QULANSRAI (CDNYL KPYVSC DCPRUL QULANSRAI (CDNYL KPYV)	327 309 275
DEEVC. ECNLKACSSNGRGELOTG DKEVVANGREGGIGVSNGRGELOTG DKEVV.SWC.DSVVTANGKGULOTA DKEVSGRECNLVVNGATRSMKNSGRGELGSC DKEVSSWG ESRUSANGKGULGEA	g svcgrrr ned v CDAVGSTAVVAVVTPEKIV SEAAGSTAVVAVIAPDKIV CDSVGSTAVVSVITPDKIV CDAVGSTAVVSVVTPEKI CDSVGSTAVVSVVTPEKI	P h VSNCGDSRAVLCRSGAAVF VANCGDSRAVLCRNGKAFFI VSNCGDSRAVLCRNGKAFV VSNCGDSRAVLCRNGVAFFI VSNCGDSRAVLCRNGVAFFI	V dghgcshva c V SSD+KPDRPDELVRI EAAGGRVI YV SVD+KPDRPDELNRI CEAGGRVI YV STD+KPDRPDELDRI EGAGGRVI YV SVD+KPDRPDELI DRI EGAGGRVI YV SVD+KPDRPDELI DRI CAGGRVI VV	W T m DCARVLCVLANSRAICDNYLKFYVIS DCFRVLCVLANSRAICDNYLKFYVSC DCFRVLCVLANSRAICDNYLKFYNSC DCARVLCVLANSRAICDNYLKFYNSC DCARVLCVLANSRAICDNYLKFYNSC	327 309 275 306 287
DEEVG. EGNLKACSSN GRGELGTE DKEVANGREGGIGVSN GRGELGTE DKEVV.SMG. DSVITAN GRGLGTE DKEVSGRECALVVNGATRSNKNSGRGELGSE DKEVV.SMG. ESVVSAN GRGLGEGE DKEVV.SMG. ESVVSAN GRGLGEGE	g SVCgrrr ned V CDAVGSTAV AVV TPEKI V SEAAGSTAVAVI APDKI V CDSVGSTAVVSVI TPDKI V CDAVGSTAVVSVI TPDKI V CDAVGSTAVVSVI TPDKI V CDAVGSTAVVSVI TPDKI V	P h VSNCGDSRAVLCRSGAAVF VANCEDSRAVLCRNGKAFF VSNCGDSRAVLCRNGKPVF VSNCGDSRAVLCRNGKPVF VSNCGDSRAVLCRNGKVF	V dghgcshva c V SSD+KPDRPDELVRI EAAGGRVI YV SVD+KPDRPDELDRI EAAGGRVI YV STD+KPDRPDELDRI EAAGGRVI YV SVD+KPDRPDELDRI EAAGGRVI YV STD+KPDRPDELJRI EAAGGRVI YV STD+KPDRPDELJRI EAAGGRVI YV	W T m DCARVLCVLANSRAI CDNYLKFYVI S DCFRVLCVLANSRAI CDNYLKFYVI S DCFRVLCVLANSRAI CDNYLKFYVSO DCRVLCVLANSRAI CDNYLKFYVI P DCFRVLCVLANSRAI CDNYLKFYVI S DCFRVLCVLANSRAI (DNYLKFYVI S DCFRVLCVLANSRAI (DNYLKFYVI S DCFRVLCVLANSRAI (DNYLKFYVI S	327 309 275 306 287 297
DEBVC. EGNLKACSSN	g svogrrr ned v CDAVGSTAV AVVTPEKI V SEAGSTAV AVVTPEKI V CDSVGSTAV SVITPEKI CDSVGSTAV SVITPEKI CDSVGSTAV SVITPEKI V CDAVGSTAV SVITPEKI V CDAVGSTAV SVITPEKI V CDAVGSTAV SVITPEKI V	P h NECCOSRAVLCRSCAAVF VENCCOSRAVLCRNCKAFF VENCCOSRAVLCRNCKAFF VENCCOSRAVLCRNCVAIF VENCCOSRAVLCRNCVAIF VENCCOSRAVLCRNCVAIF	v dghgoshva c v SEDHKPDRPDEL NI EANGENI YY SYDHKPDRPDELNI CEACENI YY SYDHKPDRPDELNI CEACENI YY SYDHKPDRPDELNI CEACENI YY SYDHKPDRPDELNI CEACENI YY SEDHKPDRPDELNI CEACENI YY SEDHKPDRPDELNI CEACENI YY SEDHKPDRPDELNI CEACENI YY	W T m DC4RVLCVLANSRAICDNYLKFYVI S DCFRVLCVLANSRAICDNYLKFYVSO DCFRVLCVLANSRAICDNYLKFYVSO DCFRVLCVLANSRAICDNYLKFYVSO DCFRVLCVLANSRAICDNYLKFYV SO DCFRVLCVLANSRAICDNYLKFYV SO DCFRVLCVLANSRAICDNYLKFYV SO DCFRVLCVLANSRAICDNYLKFYV SO	327 309 275 306 287 297 297
DEBVC, EGNLKACSSN, GRGEUTE DREVVAWGREGGIGVSN, GRGEUTE DREVV.SVC, DSVVTAN, GRGEUTE DREVSGRECNLVVKGATRSKRSGRGEUGG DREVV.SVG, SVVSAN, GRGEUGG DREVV.SKG, SVVSAN, GRGEUTE DREVS, RVSRSSVSAN, GRGEUTE DREVS, RVSRSSVSAN, GRGEUTE	g svcgrrr med v CDAVGSTAVCAVVTPEKI v SEAAGSTAVAVI APDKI v CDSVCSTAVSVI TPDKI v CDAVGSTAVSVI TPDKI v CDAVGSTAVSVI TPDKI v CDAVGSTAVSVI TPDKI v CDAVGSTAVSVI TPDKI v CDAVGSTAVSVI TPDKI v CDAVGSTAVSVI TPDKI v	P h NGCCDSRAVLCRSCAAVE CCCDSRAVLCRVCKAFF VSCCDSRAVLCRVCKAFF VSCCDSRAVLCRVCKAFV VSCCDSRAVLCRVCKAFV VSCCDSRAVLCRVCKAFV VSCCDSRAVLCRVCKAFV VSCCDSRAVLCRVCKAFV VSCCDSRAVLCRVCKAFV	v dghgcahva c v SGD+K-PDR-DEL M EA-GGRVI YV SJD+K-PDR-DEL N EG-SGRVI YV SJD-K-PDR-DEL N EG-SGRVI YV SJD-K-PDR-DEL N EG-SGRVI VV	W T M CGARVL GULANSRAI (CDNY KPYV) S CGRV GULANSRAI (CDNY KPYS) CGRV GULANSRAI (CDNY KPYS)	327 309 275 306 287 297 297 304
DEBVC. EGNLKACSSN	g svorrr ned v CDAVGSTAV GAV TPEKI v SEAGCTAV GAV TPEKI v CDAVGSTAV SVI TPEKI v CDAVGSTAV SVI TPEKI v CDAVGSTAV SVI TPEKI v CDAVGSTAV GAV TPEKI v SDAVGSTAV GAV TPEKI v SDAVGSTAV GAV TPEKI v	P h SNCCDSRAVLCRSCAAVE VNCCDSRAVLCRCKAFE NACCDSRAVLCRCKAFF SNCCDSRAVLCRCKFVF VNCCDSRAVLCRCKFVF VNCCDSRAVLCRCKFVF VNCCDSRAVLCRCKAFFF VNCCDSRAVLCRCKAFFF SNCCDSRAVLCRCKAFFF	v dghgcshva c v SGD+K+DRPDEL NEI EAN GGNV I W SJD+K+DRPDEL NEI GENGENVI W SJD+K+DRPDEL DE EGNGGNV I W SJD+K+DRPDEL NEI GENGGNVI W SJD+K+DRPDEL NEI GENGGNVI W SD+K+DRPDEL NEI GENGGNVI W SJD+K+DRPDEL NEI EEN GENGGNVI W SJD+K+DRPDEL NEI EEN GENGGNVI W	W F M DEARVL QLANSRAI CDNY KPYVI S DERRUG QLANSRAI CDNY KPYVSO DERRUG QLANSRAI CDNY KPYVSO	327 309 275 306 287 297 297 304 327
DEBVC. ECNLKACSSN	g svcgrrr med v CDAV CETAV CAVV TPEKI V SEA CETAV SVI TPEKI V CDSVCSTAV SVI TPEKI V CDSVCSTAV SVI TPEKI CDAVCSTAV SVI TPEKI V CDAVCSTAV SVI TPEKI V SDAVCSTAV SVI TPEKI V SDAVCSTAV AVV TPEKI V CDAVCSTAV SVI TDEKI V CDAVCSTAV SVI TPEKI V CDAVCSTAV SVI TTEKI	P h VENCEDSRAVLCRSCAAVE NCCDSRAVLCRNCKAFF NCCDSRAVLCRNCKAFF NCCDSRAVLCRNCKFVF VENCEDSRAVLCRNCKFVF VENCEDSRAVLCRNCKAFF VENCEDSRAVLCRNCKAFF VENCEDSRAVLCRNCKAFF VENCEDSRAVLCRNCKAFF	dghgcshva c v sock-kporpolu Ki BANGGNI yv sock-kporpolu Ki BANGGVI yv sock-kporpolu Ki BANGVI yv sock-kporpolu VV sock-kporpolu Ki BANGVI yv sock-kporpolu Ki BANG	W T TO CGRAVL GULANSRAI CDN YL KFYVI S CGRAV GULANSRAI CDN YK KFYVI S CGRAV GULANSRAI CDN YKFYVI S	327 309 275 306 287 297 304 327 299
DENC. EGNLKACSBN	9 SVC9TFT REG V CDAVGCTAVGAV, TPEKI V SEACCTAVGAV APCKI DEVCETAVGAV APCKI CDAVGCTAVGAVI FEDRI V CDAVGCTAVGAVI FEDRI V COAVGCTAVGAVI FEDRI V COAVGCTAVGA	P RANCEDSRAVE CRESCAAVE WINCEDSRAVE CRESCAAV	v dghgcanva c v SEDHKPDRPDEL NE EANGENI YV SUDHKPDRPDEL NE GEACGNI YV SUDHKPDRPDEL NE BACGGNI YV	W T M CCARVL GULANSRAI (CDNY KPYV) S CEFRU GULANSRAI (CDNY KPYV) CERVI (CULANSRAI (CDNY KPYV)	327 309 275 306 287 297 304 327 269 324
DEEVC. EGNLKACSSN. GREETE NEVVAMCREGGICVSN. GREETE NEVV, SMG. DSVITAN. GROETE DKEVSGRECN.VVNCPTRSNKNGGGELGSG DKEVSGRECN.VVNCPTRSNKNGGGELGSG DCEV-, RRSCSNCTFT. GREETE DCEVC. EGNLVACGFN. GRGETE DCEVC. EGNLVACGFN. GRGETE DCEVC. EGNLVACGFN. GRGETE DCEVC. LENLVACGFN. GRGETE DCEVC. LENLVACGFN. GRGETE DCEVC. INSKAN-SSA. GRGETES DCEVC. INSKAN-SSA. GRGETES DCEVC. INSKAN-SSA. GRGETES DCEVC. INSKAN-SSA. GRGETES	9 SVC9TFT MED V CDAVGETA CANATPER SARGSTA VANATPER UDSVCETA VSVITPCH CDAVGETA VSVITPCH CDAVGETA VSVITPCH CDAVGETA VSVITPCH SDAVGETA VANATPER SDAVGETA VANATPER CDAVGETA VANATPER CDAVGETA VANATPER	P h NACCEDSRAUL CREGARY WACCEDSRAUL CREGARY NACEDSRAUL CREGARY	V dghgcshva c V SGD+K+DRPDELINE (EA-GGRVI YV SJD+K+DRPDELINE (EA-GGRVI YV)	W T 00 CGRRUL GULANSRAI CENTU KFYVI S DGRRUG GLANSRAI CENTU KFYVI S	327 309 275 306 287 297 304 327 269 324 329
DEBVC. ECNLKACSSN. GROEDT KEVVAMOREGO (CVSN. GROEDT DKEVV. SWG. DSVVTAN. GROEDT DKEVV. SWG. DSVVTAN. GROEDT DKEVV. SWG. ESVVSAN. GROEDT DKEVV. SWG. ESVVSAN. GROEDT DKEV. RNSCSNCTFT. GROEDT DKEVS. RVSNESVESAN. GROEDT DCVG. ECNLVACGFN. GROEDT DCVG. ECNLVACGFN. GROEDT DCVG. ECNLVACGFN. GROEDT DCVG. ECNLVACGFN. GROEDT DCVG. CONLVACGFN. GROEDT DCVG. CONLVACGFN. GROEDT DCVG. CONLVACGFN. GROEDT	9 SUCGTYPT MED V QEAVGSTAVGAVGAPGAV QEAVGSTAVGAVGAPGAV QEAVGSTAVGAVGAPGAV CDAVGSTAVGAVGAPGAV CDAVGSTAVGAVGAPGAV CDAVGSTAVGAVGAPGAV CDAVGSTAVGAVGAPGA CDAVGSTAVGAVGSGAV CDAVGSTAVGAVGSGAV CDAVGSTAVGAVGSGAV	P Reception of the second of	V dghgcshva c V SOLKEDRPDEL VR EAAGGRVI YV SOLKEDRPDEL VR IGAGGRVI YV SOLKEDRPDEL VR IGAGGRVI YV SOLKEDRPDEL IM IGAGGRVI YV SOLKEDRPDEL IM IGAGGRVI YV SOLKEDRPDEL IM IGAGGRVI YV SOLKEDRPDEL IM IGAGGRVI YV SOLKEDRPDEL VR IGAGGRVI YV SOLKEDRPDEL IM IGAGGRVI YV	W T TO CERTUL GULANSRAILCENTUL KEYVI S CERTUL GULANSRAILCENTUL KEYVI S CERTUL GULANSRAILCENTUL KEYVI SE CERTUL GULANSRAILCENTUL KEYVI SE CERTUL GULANSRAILCENTUL KEYVI S CERTUL GULANSRAILCENTUL KEYVI S	327 309 275 306 287 297 304 327 269 324 329 304
DEBVC. EGNLKACSSN. GREETE DKEVANCREGGIGVGN. GREETE DKEVV.SWG.DSVTAN. GROETE KEVSGRECALVNGATRSNKNSGREGGSG DKEVV.SWG.ESVVSN. GROETE DGEV- RRGCNCTFT. GREETE DKEVANS.EGGCVGN. GREETE DKEVANS.EGGCVGN. GREETE DKEVANS.EGGCVGN. GREETE DKEV.LLS.ESVVSN. GREETE DGC.C.EGNLVACGFN. GREETE DKEVANS.EGGCVGN. GREETE DKEVANS.EGGCVGN. GREETE DKEVANS.EGGCVGN. GREETE	9 SVG9TFT MED V CDAVGSTA CANVTPER CDSVGSTA VSVTFBC VDSVGSTA VSVTFBC CDSVGSTA VSVTFBC VDSVGSTA VSVTFBC VDAVGSTA VSVTFBC VDAVGSTA VSVTFBC VDAVGSTA VSVTFBC VDAVGSTA VSVTFBC VDAVGSTA VSVTFBC VDAVGSTA VSVTFBC VDAVGSTA VSVTFBC	P R NACEDSRAUL CRESCAAVE NACEDSRAULCRICK CAPEN NACEDSRAULCRICK CAPEN NACEDSRAULCRICK OF A NACEDSRAULCRICK OF A NACEDSRAUCRICK OF A NA	V dghgcanva c V SEDHKPDRPDEL VR EANGERVI YV SUDHKPDRPDEL NG EGACGRVI YV SUDHKPDRPDEL IN EGACGRVI YV	W T M CGARVL GULANSRAI (CDNY KPYV) S CGFRVL GULANSRAI (CDNY KPYV) G CGFRVL GULANSRAI (CDNY KPYV) C CGFRVL GULANSRAI (CDNY KPYV) C CGFRVL GULANSRAI (CDNY KPYV) S CGFRVL GULANSRAI (CDNY	327 309 275 306 287 297 304 327 269 324 329 324 329 304 306
DEBVC. EGNLKACSSN	9 SVC9777 MED V CDAVGETA CANATPEEN SEAACETA CANATPEEN SEAACETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN DAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN CDAVGETA CANATPEN COALCOLORIGA CANATPEN COALCOLORIAN CANATPEN COALCOLORIAN CANATPEN COALCOLORIAN CANATPEN COALCOLORIAN CANATPEN COALCOLORIAN CANATPEN COALCOLORIAN CANATPEN	P Recebsravices Rec	dghgcshva c sockeporpolu Ki BANGGNI yv storkeporpolu Ki BANGGNI yv storkepolu BANGGNI yv storkepolu HABANGGNI yv storkepolu HABANGNI y	W TO CGRAVL GULANSRAI CDN/U KPYVI S CGRAVL SU ANSRAI CDN/U KPYVI S CGRAVI SU ANSRAI CDN/U KPYVI S CGRAVI SU ANSRAI CDN/U KPYVI S CGRAVI SU ANSRAI CDN/U K	327 309 275 306 287 297 304 327 269 324 329 304 329 304 306 310
DEEVC. EGNLKACSSN. GREETER DKEVANCREGGIGVGN. GREETER DKEV, SMG.DSVTAN. GROETER DKEV, SMG.DSVTAN. GROETER DKEV, SMG.ESVVSN. GROETER DGEV. RRSCSKTFT. GROETER DKEVAMS.EGGGVGS. GROETER DKEVAMS.EGGGVGS. GROETER DDV.LLS.ESVVSN. GROETER DDV.LLS.ESVVSN. GROETER DDV.L.S.ESVVSN. GROETER DDV.L.S.ESVSN. GROETER DDV.L.S.ESVSN. GROETER DEV.C.EGN.VACFR. GROETER DEV.TATT.CTVAGAN. GROETER GROETER	9 SVC9TFF MED V 26A/CCTAVAVTEEX 26A/CCTAVAVAPCN CDSVC5TAVSVTEEX 10DSVC5TAVSVTEEX 10DSVC5TAVSVTEEX 10DSVC5TAVSVTEX 10DA/CCTAVAVTEX 10DA	P R R R R R R R R R R R R R	V dghgcanva c V SGD+K-PDRPDEL NG EAAGGRVI YV SJD+K-PDRPDEL NG EAAGGRVI YV SJD+K-PDRPDEL NG EAGGRVI YV	W T M CGARVL GULANSRAI (CDNY KPYV) S CGRVL GULANSRAI (CDNY KPYV) C CGRVL GULANSRAI (CDNY KPYV) C CGRVL GULANSRAI (CDNY KPYV) C CGRVL GULANSRAI (CDNY KPYV) C CGRVL GULANSRAI (CDNY KPYV) S CGRVL GULANSRAI (CDNY K	327 309 275 306 287 297 304 327 269 324 329 304 329 304 306 310 299
DEEVC. EGNLKACSSN. GREETE REVAMERECEICVEN. GREETE NEVV.SMC.DSVTAM. GREETE NEVV.SMC.DSVTAM. GREETE NEVV.SMC.SSVVAA. GREETE DEVC.RRSCNCTFT. GREETE NEVV.SMC.ESVVAA DEV.RRSCNCTFT. GREETE DEVC. EGNLVACFN. GREETE DEVC. EGNLVACFN. GREETE DEVC. EGNLVACFN. GREETE DEVC. EGNLVACFN. GREETE DEVC. EGNLVACFN. GREETE DEVC. EGNLVACFN. GREETE DEVC. SWASSETVT. GREETE DEVC.S.SWASSETVT. GREETE DEVC.S.SWASSETVT. GREETE	9 SVC9777 MED V CDAVGSTAVAVTPEM SEARCSTAVAVA USACSTAVASVTPCAV CDAVGSTAVSVTPEM UDAVGSTAVSVTPEM UDAVGSTAVAVTPEM SDAVGSTAVAVTPEM SDAVGSTAVAVTPEM SDAVGSTAVAVTPEM SDAVGSTAVAVTPEM CDAVGSTAVAVTPEM CDAVGSTAVAVTPEM CDAVGSTAVAVTPEM CDAVGSTAVAVTPEM	P Reccessrave conservations Reccessrave conser	Geherona Control Contr	W TO CGRRVL GULANSRAI CENYU KEYVI S DGRVU GULANSRAI CENYU KE	327 309 275 306 287 297 304 327 269 324 329 304 306 310 299 326
DEBVC. ECNLKACSSN. GREETE KEVVAWCREGGIGVSN. GREETE DKEVV.SWG.DSVVTAN. GROETE DKEVV.SWG.DSVVTAN. GROETE DKEVV.SWG.ESVVSAN. GROETES DKEV.SWG.ESVVSAN. GROETES DKEV.SWG.ESVVSAN. GROETES DKEVV.SWG.EGGGVS-GROETEG DCBVC. ECNLVACGFN. GROETEG DCBVC. ECNLVACGFN. GROETEG DCBVC. ECNLVACGFN. GROETEG DCBVC. ECNLVACGFN. GROETEG DCBVC. SWNSSSCTVT. GROETEG DCBVC. SWNSSSCTVT. GROETEG DCBVC. ECNLVACGFN. GROETEG DCBVC. SWNSSSCTVT. GROETEG DCBVC. SWNSSSCTVT. GROETEG DCBVC. SKNSSSCTVT. GROETEG	9 SVC9777 MEX V CDAVGSTAVGAV APDA SEACSTAVGAV APDA DEVGTAVGAV APDA LDAVGSTAVGAV APDA LDAVGSTAVGAV TPDA LDAVGSTAVGAV TPDA DAVGSTAVGAVTPDA LDAVGSTAVGA	P R R R R R R R R R R R R R	V dghgcshva c V SOL+KPDRPDEL Ni EANGGNI YY SID-KPDRPDEL Ni EANGGNI YY SID-KPDRPDEL Ni EANGGNI YY SID-KPDRPDEL Ni EAGGGNI YY SID-KPDRPDEL NI EAGGNI YY	W T 00 CGRVL GULANSRAI CDNV KPYVI S CGRVL GULANSRAI CDNV KPYVSC CGRVL GULANSRAI CDNV KPYVSC CGRVL GULANSRAI CDNV KPYV SC CGRVL GULANSRAI CDNV KPYV SC	327 309 275 306 287 297 304 329 304 329 304 306 310 299 326 304
DEEVC.       EGNLKACSSN.       GREENT GREENT GREENT GREENT GREENT WARDEREGT (2V.S.).       GREENT GREENT WARDERSKAS GREENT GREENT GREENT WARDERSKASSN.         OKEV.SWG.ESVVSN.       GREENT	9 SVG9TFF RED V CDAVGSTA KANATPER CDAVGSTA KANATPER CDSVGSTA VSVTFBC/ CDSVGSTA VSVTFBC/ CDAVGSTA VSVTFBC/ CDAVGSTA VSVTFBC/ VDAVGSTA VSVTFBC/ VDAVGSTA VSVTFBC/ VDAVGSTA VSVTFBC/ DAVGSTA VSVTFBC	P R R R R R R R R R R R R R	V dghgcanva c V SOHKEDRPDEL NG EAAGGNU YV SOHKEDRPDEL NG EAAGGNU YV	W T M CCARVL QL LANSRAI (CDNYL KPYV) S CGRVL CQL LAN	327 309 275 306 287 297 304 327 304 329 304 306 310 299 326 304
DEBUC, EGNLKACSSN. GROENT KEVAMGREGGICVSN. GROENT DKEVSAMGREGGICVSN. GROENT DKEVSGRECN.VVNAPTRSNKSGROENG GROV, SWE.SVVSAN. GROENG DCBV, SWE.SVVSAN. GROENG DCBV, RRSCSNCTFT. GROENT DCBVC, EGNLVACGPN. GROENT DCBVC, EGNLVACGPN. GROENT DCBVC, EGNLVACGPN. GROENT DCBVC, EGNLVACGPN. GROENT DCBVC, EGNLVACGPN. GROENT DCBVC, EGNLVACGPN. GROENT DCBVC, CLVACGPN. GROENT DCBVC, SWNSSSCTVT. GROENT DCBVC, SWNSSSCTVT. GROENT DCBVC, SWNSSSCTVT. GROENT DCBVC, SWNSSSCTVT. GROENT DCBVC, GRULVACGPN. GROENT DCBVC, SWNSSSCTVT. GROENT DCBVC, GRULVACGPN. GROENT DCBVC, CLVACGPN.	9 SVC9777 MED V CDAVGSTAV GAVATEEM SEAACSTAV GAVA APDOI 105% CST-VX SVT FEAA 105% CST-VX SVT FEAA 105% CST-VX SVT FEAA 105% CST-VX SVT FEAA 105% CST-VX GAV TEDOI 105% CST-VX G	P R Records and the second se	V dghgcshva c V SOLKEDRPDELVR EARGENU YV SOLKEDRPDELVR EARGENU YV SOLKEDRPDELVR EARGENU YV SOLKEDRPDELIR EARGENU YV	W TO CGRAVL GULANSRAI CDNYL KPYVI S CGRAVL GULANSRAI CDNYL KPYVI S CGRAVL GULANSRAI CDNY KPYVI S CGRAVL SULANSRAI CDNY K	327 309 275 306 287 297 304 327 269 324 329 304 306 310 299 326 304
DEEVC. EGNLKACSSN. Greeter KEVVANCREGGIGVGN. Greeter KEVV.SNG.DSVTAN. Greeter KEVV.SNG.DSVTAN. Greeter KEVV.SNG.ESVVSN. Greeter DEVC.RRGCNCTFT. Greeter Greeter DEVC.RRGCNCTFT. Greeter Greeter DEVC.EGNLVACGPN. Greeter DEVC.EGNLVACGPN. Greeter DEVC.EGNLVACGPN. Greeter DEVC.EGNLVACGPN. Greeter DEVC.EGNLVACGPN. Greeter DEVT.VT.GCTARB.GREETE GREVANS.EGGGCVSI. Greeter GREVANS.EGGGCVSI. Greeter GREVANS.EGGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. Greeter GREVANS.EGGCVSI. GREVER GREVANS.EGGCVSI. GREVER GREV	9 SVC9777 MEX V 26A/CETA/CAV/APE/01 26A/CETA/CAV/APE/01 26A/CETA/CAV/APE/01 CDA/CETA/CAV/APE/01 CDA/CETA/CAV/TPC/01 CDA/CETA/CAV/	P R Records and the second se	V dghgcshva c V SOLKEDRPDEL N ( EARGEN V YV SOLKEDRPDEL N ( EARGEN V YV) SOLKEDRPDEL N ( EARGEN V YV) SOLKEDRPDEN ( E	W T M CGRVL GULANSRAI (CDNV KPYV) S CGRVL GULANSRAI (CDNV KPVV S CGRVL GULANSRA	327 309 275 306 287 297 304 327 269 324 329 304 306 310 299 326 304 423
DEBUC, EGNLKACSSN. GROUTE REVAMCRECCICVCN. GROUTE NEVV.SWC.DSVTAN. GROUTE NEVV.SWC.DSVTAN. GROUTE NEVSCRECALVNGATRSIKKSGGLGSC DEV.RRCSNTFT. GROUTE NEVS.RWSRESSAN. GROUTE DEV.RRCSNTFT. GROUTE DEV.RRSSVSAN. GROUTE DEV.RRSSVSAN. GROUTE DEV.LS.ESVVSAN. GROUTE DEV.S.ESVVSAN. GROUTE DEV.LS.ESVVSAN. GROUTE DEV.	9 SVC9777 MED V CDAVGETA KANATPER BAGCSTA VANATPER CDSVC974 SV1TPCV CDAVGETA VSV1TPCV CDAVGETA VSV1TPCV CDAVGETA VSV1TPCV SDAVGETA VSV1TPCV SDAVGETA VSV1TPCV SDAVGETA VSV1TPCV CDAVGETA VSV1TPCV SDAVGETA VSV1TPCV CDAVGETA VSV1TPCV SDAVGETA VSV1TPC	P NCCEDSRAULCR GAAP NCCEDSRAULCR GAAP NCCEDSRAULCR GAP NCCEDSRAULCR GAP NCCESSAULCR GAP	V dghgcshva C V SCD+KPDRPDELVR (EAACGRVI YV SD-KPDRPDELVR (EAACGRVI YV SD-KPDRPDELVR (EAACGRVI YV SD-KPDRPDELLR (EAACGRVI YV SD-KPDRPDELLR (EAACGRVI YV SD-KPDRPDELLR (EAACGRVI YV SD-KPDRPDELLR (EAACGRVI YV SD-KPDRPDELVR (EAACGRVI YV SD-KPDRPDELVR (EAACGRVI YV SD-KPDRPDELLR (EAACGRVI YV) SD-KPDRPDELLR (EAACGRVI YV) SD-KPDRPDRPDELLR (EAACGRVI YV) SD-KPDRPDRPDELLR (EAACGRVI YV) SD-KPDRPDRPDELLR (EAACGRVI YV) SD-KPDRPDRPDELLR (EAACGRVI YV) SD-KPDRPDRPDL (E	W T TO CGRRVL GULANSRAI CENYU KYVI S DGRVU GULANSRAI CENYU KYVI S DGRFU GULANSRAI CENYU KYVI	327 309 275 306 287 297 304 327 269 324 329 304 306 310 299 326 304 423 406
DEEVC. EGNLKACSSN. Greet off KEVVAWGREGGI CVSN. Greet off OKEVCSNC, SWG, DSVVTAN. Greet off OKEVCSNC, SWG, DSVVTAN. Greet off OKEVCSNC, SESVSAN. Greet off OKEVCSNC, SESVSAN. Greet off DEVCVAWS, EGGCOVSN. Greet off DEVCVAUS, EGGCOVSN. GREET O	9 SVC9777 MEX V 284/CETAV CAVI A PED 0 284/CETAV CAVI A PED 0 CDAV CETAV CAVI A PED 0 CDAV CETAV CAVI A PED 0 CDAV CETAV CAVI FPC 0 SC 0 CAVI CETAV CAVI FPC 0 SC 0 CAVI CETAV CAVI FPC 0 SC 0 CAVI CETAV CAVI FPC 0 SC 0 COV FPC 0 COV CETAV CAVI FPC 0 SC 0 COV CETAV CAVI FPC 0 COV CETAV CAVI FPC 0 COV CETAV CAVI FPC 0 SC 0 COV CETAV CAVI FPC 0 COV CETAV CETA	P R Records and the second se	V dghgcahva c V SOLKKPDRPDELVR EARGENVI YV SODKKPDRPDELVR EARGENVI YV SOLKPDRPDELLR EGAGRVI YV SOLKPDRPDELLR SOLVSVI VVDI SOLKPDRPDELLR EGAGRVI YV SOLKPDRPDELLR EGAGRVI YV SOLKPDRPDELLR EGAGRVI YVDI SOLKPDRPDELLR EGAGVVI YVDI SOLKPDRPDRPDELLR EGAGRVI YVDI SOLKPDRPDRPDELLR EGAGVVI VVDI SOLKPDRPDRPDELLR EGAGVVI VVDI SOLKPDRPDRPDRPDELLR EGAGVVI VVDI SOLKPDRPDRPDRPDVVVVDI SOLKPDRPDRPDRVVVVDI SOLKPDRPDRPDVVVVDI SOLKPDRPDRPDRVVVVDI SOLKPDRPDRPDRVVVVDI SOLKPDRPDRVVVVDI SOLVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVDI SOLVVVVVVDI SOLVVVVVDI SOLVVVVVVDI SOLVVVVVVDI SOLVVVVVVDI SOLVVVVVVDI SOLVVVVVVDI SOLVVVVVVVDI SOLVVVVVVVDI SOLVVVVVVVDI SOLVVVVVVVVDI SOLVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVV	W T M CGRVL GULANSRAI CDNU KPYVI S CGRVL GULANSRAI CDNU KPYVSC CGRVL GULANSRAI CDNU KPYCSC CGRVL GU	327 309 275 306 287 297 304 329 324 304 306 310 299 326 304 423 304
DEEVC.         EGNLKACSSN.         GRELOTE           VKEVANCRECCI (VCN.         GRELOTE           VKEV, SWC. DSVTAN.         GRELOTE           VKEV, SWC. DSVTAN.         GRELOTE           VKEVSKA.         GRELOTE           VKEVSKA.         GRELOTE           DEV.         RRECSVTAN.           DEV.         RRESSNETAN.           DEV.         RRESSNETAN.           DEV.         RRESVESAN.           DEV.         RRESVESAN.           DEV.         RRESVESAN.           DEV.         RRESVESAN.           DEV.         REGECEVEL.           DEV.         REGECEVEL.           DEV.         LUS.ESVVSAN.           DEV.         LUS.ESVSAN.           DEV.         LUS.ESVVSAN.           DEV.         LUS.ESVVSAN.           DEV.         LUS.ESVSAN.           DE	9 SVC9TFT MED V CDAVGETA CANATPER BARGSTA VANATPER CDSVGETA VSVITEPON CDSVGETA VSVITEPON CDAVGETA VSVITEPON DOAVGETA VSVITEPON	P R R R R R R R R R R R R R	V dghgcanva c V SGD+K-PDRPDEL N K EA/GGN/ YV SJD+K-PDRPDEL N EA/GGN/ YV SJD+K-PDRPDR/ SJD/ VV SJD+K-PDRPDR/ SJD/ V	W T M CCARVL GULANSRAI (CDNYL KPYV) S CGRVU GULANSRAI (CDNYL	327 309 275 306 287 297 304 329 324 329 304 306 310 299 326 304 423 304 423 362 399
DEEVC.         EGNLKACSSN.         GR GELGTE           MELVAMGREGEI EVSN.         GR GELGTE           MEV.SMG.DSVVTAN.         GR GELGTE           MEV.SMG.DSVVTAN.         GR GELGTE           MEV.SGRECN.VVNCATRSHKNSGR GELGSE         GR GELGTE           DEV.SR.SESVSAN.         GR GELGTE           DEV.S.RNSESVSAN.         GR GELGTE           DEV.S. ENN.VACEPN.         GR GELGTE           DEV.V.LSEN.VACEPN.         GR GELGTE           DEV.S. ENN.VACEPN.         GR GELGTE           DEV.V.VED.VACEPN.         GR GELGTE           DEV.V.VED.VACEPN.VACEPN.         GR GELGTE           DEV.V.VED.VACEPN.VACEPN.         GR GELGTE           DEV.V.VED.VACEPN.VACEPN.         GR GELGTE           DEV.V.VED.VACEPN.VACEPN. </td <td>9 SVC9777 MEX V 9 SVC9777 MEX V 9 SAACETAV AVV FEEM 10 SCATAV SVT FECH 10 CAVESTAV S</td> <td>P R Receiled and a second a second and a second a second and a second and a second and a second and a second a</td> <td>V dghgcshva c V SOLKKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELIR GAAGGRVI YV SOLVARDRPDELIR GAAGGRVI YV SOLVARDRPDING GAAGGRVI YV SOLVARDRPDING</td> <td>W T TO CGRVL GULANSRAI CDNYL KPYVI S CGRVL GULANSRAI CDNYL KPYVI S CGRVL GULANSRAI CDNY KPYVI S CGRVL GULANSRAI CDNY KPYVI S CGRVL GULANSRAI CDNYL KPY</td> <td>327 309 275 306 287 297 304 329 304 329 304 329 304 306 310 299 306 310 299 306 310 299 306 304 306 306 310 299 326 306 306 329 329 329 329 329 329 329 329 329 329</td>	9 SVC9777 MEX V 9 SVC9777 MEX V 9 SAACETAV AVV FEEM 10 SCATAV SVT FECH 10 CAVESTAV S	P R Receiled and a second a second and a second a second and a second and a second and a second and a second a	V dghgcshva c V SOLKKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELIR GAAGGRVI YV SOLVARDRPDELIR GAAGGRVI YV SOLVARDRPDING GAAGGRVI YV SOLVARDRPDING	W T TO CGRVL GULANSRAI CDNYL KPYVI S CGRVL GULANSRAI CDNYL KPYVI S CGRVL GULANSRAI CDNY KPYVI S CGRVL GULANSRAI CDNY KPYVI S CGRVL GULANSRAI CDNYL KPY	327 309 275 306 287 297 304 329 304 329 304 329 304 306 310 299 306 310 299 306 310 299 306 304 306 306 310 299 326 306 306 329 329 329 329 329 329 329 329 329 329
DEEVC.         EGNLKACSSN.         GR GELGTE           VKEVANCRECCICVCN.         GR GELGTE           VKEVANCRECCICVCN.         GR GELGTE           VKEVSNEL.         GR GELGTE           VKEVSKEL.         GR GELGTE           VKEVSKEL.         VNASTREMKSGR GELGGE           DERV.         RRSCSNTFT.         GR GELGTE           DERV.         RRSCSNTFT.         GR GELGTE           DERV.         RRSCSNTFT.         GR GELGTE           DERV.         RRSCSNTFT.         GR GELGTE           DEV.         EGNLVACEPN.         GR GELGTE           DEV.         LSK.         SEVSAN.           DEV.         LNACEPN.         GR GELGTE           DEV.         LNACEPN.         GR GELGTE           DEV.         LNACEPN.         GR GELGTE           DEV.         LNACEPN.         GR GELGTE           DEV.         LSEVSESCIT.         GR GELGTE           DEV.         LEGGECVS. <t< td=""><td>9 SVC9777 MEX V 284ACSTAVCAVATEEM 384ACSTAVCAVATEM 185ACSTAVCAVATEM CDAVCSTAVCAVATEM COVENCICACCASCSE COVENCICACCA</td><td>P R SNCCDSRAULCRCAAP NCCDSRAULCRCAAP NCCDSRAULCRCAP NCCDSRAU NCCDSRAU NCCDSRAULCRCAP NCCDSRAULCRCAP NC</td><td>V dghgcahva c V SOLKKPDRPDEL Ni EAAGGNU YV SODKKPDRPDEL Ni EAAGGNU YV SODKRPDRPDEL NI EAAGGNU YV SODKRANGNU AAGSONVSV VD SODKRPDRPDEL NI EAAGGNU YV SODKRANGNU AAGSONVSV VD SODKRANGNU AAGSONVSV VD SODKRANGNU AAGSONVSV VD SODKRANGNU AAGSONVSV VD SODKLAAGSONVSV VD</td><td>W T M  CGRVLGULANSRAILCENYU KYYVI S  CGRVLGULANSRAILCENYU KYVI S  CGRVLANSRAILCENYU KYVI S  CGRVLANSRAILCENYU KYVI S  CGRVLAN</td><td>327 309 275 306 287 297 304 329 304 329 304 329 304 306 310 299 306 310 299 304 306 310 299 304 306 306 306 306 306 306 306 306 306 306</td></t<>	9 SVC9777 MEX V 284ACSTAVCAVATEEM 384ACSTAVCAVATEM 185ACSTAVCAVATEM CDAVCSTAVCAVATEM COVENCICACCASCSE COVENCICACCA	P R SNCCDSRAULCRCAAP NCCDSRAULCRCAAP NCCDSRAULCRCAP NCCDSRAU NCCDSRAU NCCDSRAULCRCAP NCCDSRAULCRCAP NC	V dghgcahva c V SOLKKPDRPDEL Ni EAAGGNU YV SODKKPDRPDEL Ni EAAGGNU YV SODKRPDRPDEL NI EAAGGNU YV SODKRANGNU AAGSONVSV VD SODKRPDRPDEL NI EAAGGNU YV SODKRANGNU AAGSONVSV VD SODKRANGNU AAGSONVSV VD SODKRANGNU AAGSONVSV VD SODKRANGNU AAGSONVSV VD SODKLAAGSONVSV VD	W T M  CGRVLGULANSRAILCENYU KYYVI S  CGRVLGULANSRAILCENYU KYVI S  CGRVLANSRAILCENYU KYVI S  CGRVLANSRAILCENYU KYVI S  CGRVLAN	327 309 275 306 287 297 304 329 304 329 304 329 304 306 310 299 306 310 299 304 306 310 299 304 306 306 306 306 306 306 306 306 306 306
DEEVC. EGNLKACSSN. GROUTE REVAMCRECCICVCN. GROUTE WEV.W.SMC.DSVTAM. GROUTE WEV.SWC.SSVTAM. GROUTE WEV.SWC.SSVTAM. GROUTE DEVC. RRSCNCTFT. GROUTE WEV.SWC.ESVTAM. GROUTE DEVC. RRSCNCTFT. GROUTE WEV.SWAMS.EGGEVSH. GROUTE DEVC. ECALVACEPN. GROUTE DEVC. INSKANSSA. GROUTE DEVC. INSKANSSA. GROUTE DEVC. ISSN GROUTE DEVC. GROUTAGEPN. GROUTE DEVC. ISSN GROUTE DEVC. GROUTAGEPN. GROUTAGEPN. GROUTAGEPN.	9 SVC9777 REV V CDAVGSTAVAN/TPEM SEARCSTAVAN/TPEM USACSTAVAN/TPCM CDAVGSTAVSV/TPCM CDAVGSTAVSV/TPCM CDAVGSTAVSV/TPCM SDAVGSTAVAN/TPCM SDAVGSTAVAN/TPCM SDAVGSTAVAN/TPCM CDAVGSTAVAN/TPCM SDAVGSTAVAN/TP	P RANGE STANLER GANER ACCESSRAULER GANER ACC	V G9h9Cshva C V SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDEL R EANGGRVI YV SOLKFDRPDEL R EANGGRVI YV SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDELVR EANGGRVI YV SOLKFDRPDEL R EANGGRVI YV SOLKFDRPDRPDEL R EANGGRVI YV SOLKFDRPDRPDEL R EANGGRVI YV SOLKFARDRPDEL R EANGGRVI YV SOLKFARDRPDEV R EANGGRVI	W T TO CGRAVL GULANSRAI CENYU KEYVI S CGRAVL S CGRAVI S CGRAVI S CGRAVI S CGRAVI S CGRA	327 309 275 306 287 297 324 329 324 329 304 310 299 326 304 423 406 362 399 381 400 391
DEEVC.         EGNLKACSSN	9 SVC9777 MEX V 2 SVC9777 MEX V 2 SAACSTAV GAVI A PED ( 1 V CDAV CSTAV GAVI TPC)	P R SACCEDSRAULCR GAAVE ACCEDSRAULCR GAAVE ACCEDSRAULCR GAPPE ACCEDSRAULCR GAPPE ACCEDSRAUCR GAPPE ACCEDSRAUCR GAPE ACCEDSRAUCR GAP	V dghgcahva c V SOL-K-PDR-PDELVR (EA-RGENV) YV SOD-K-PDR-PDELVR (EA-RGENV) YV SOD-K-PDR-PDELVR (EA-RGENV) YV SOD-K-PDR-PDELLR (EA-RGENV) YV SOD-K-PDR-PDR-PDR-PDR-PDR-PDR-PDR-PDR-PDR-PDR	W T TO CARVL GULANSRAI CDNU KPYVI S CARVL GULANSRAI CDNU KPYVI S	327 309 275 306 287 297 304 329 304 300 299 326 304 310 299 326 304 406 362 399 381 400 391 399
DEEVC.         EGNLKACSSN.         GR GELGTE           VKEVANCRECGI CVCN.         GR GELGTE           VKEV.         SWG. DSVTAN.         GR GELGTE           DEV.         REGOLVSN.         GR GELGTE           DEV.         RESSENSAN.         GR GELGTE           DEV.         RESSVESAN.         GR GELGTE           DEV.         RESVESAN.         GR GELGTE           DEV.         RESVESAN.         GR GELGTE           DEV.         RESVENSAN.         GR GELGTE           DEV.         LESUVAN.         GR GELGTE           DEV.         LESUVAN.         GR GELGTE           DEV.         LUACFN.         GR GELGTE           DEV.         LUACFN.         GR GELGTE           DEV.         LUACFN.         GR GELGTE           GEV.         SKGTVL         GR GELGTE           DEV.         LASSCTVT.         GR GELGTE           GV         C C LASSCTVT.         GR GELGTE           GV         C C LASSCLVVVSNNT         GR GELGTE           GV         C	9 SVC9777 MED V CDAVGETA ANV TPEM DEAVESTA ASV TPEM UDS/GETA VSV TPOM UDS/GETA VSV TPOM UDS/GETA VSV TPOM UDS/GETA VSV TPOM UDAVGETA VSV T	P NCCEDSRAULCREAN NCCEDSRAULCREAPUE NCCEDSRAULCR	V dghgcshva c V SOLKFDRPDELVR EANGGRVI YV SUD-KFDRPDELVR EANGGRVI YV SUD-KFDRPDELVR EANGGRVI YV SUD-KFDRPDELIR EGNGGRVI YV SUD-KFDRPDELIR EGNGGRVI YV SUD-KFDRPDELIR EGNGGVI YV SUD-KFDRPDELIR EGNGVI YV	W I TO CGRIPUL GULANSRAI CENNUK YVV S CGRIPUL GULANSRAI CENNUK YVVSC CGRIPUL GULANSRAI CENN	327 309 275 306 287 297 304 329 324 304 306 310 299 326 304 423 304 400 362 399 381 400 399 424
DEEVC. EGNLKACSSN	9 SVC9777 MEX V 9 SVC9777 MEX V 9 SAACTTA VAN THE M 9 SAACTTA VAN THE M 10 VC VAN THE M	P R Records and constructions	V G9h9CshV3 C V SOLKKPDRPDELVR GAAGGKVI VY SOLKPDRPDELVR GAAGGKVI VY SOLKPDRPDELVR GAAGGKVI VY SOLKPDRPDELIR GAAGGKVI VY SOLVEN SOLVEN SOLKPDRPDELIR GAAGGKVI VY SOLVEN S	W T TO CGRVL GULANSRAI CDNYL KPYVI S CGRVL GULANSRAI CDNYL KPYVI S CGRVL GULANSRAI CDNY KPYVI S CGRVL GULANSRAI CDNY KPYVI S CGRVL GULANSRAI CDNYL S CGRVL GULANSRAI CDNYL S CGRVL GULANSRAI CDNYL S CGRVL GULANS	327 309 275 306 287 297 304 329 304 329 304 300 299 326 304 423 406 362 399 381 400 391 399 381 424 366
DEEV.C. EGNLKACSSN	9 SVC9777 MEX V TPEM CDAVGSTA AAV TPEM DDAVGSTA AAV TPEM CDSVC57A SVT TPCA CDSVC57A SVT TPCA CDSVC57A SVT TPCA DDAVGSTA SVT TPCA SVT	P R SNCCDSRAULCRCAAP NCCDSRAULCRCAAP NCCDSRAULCRCAP NCCDSRAU NCCDSRAULCRCAP NCCDSRAULCRCAP NCCDSRAULCRC	V d9h9cshva c v SOL+KPDRPDELVR (EAAGGNU YV SOD-KPDRPDELVR (EAAGGNU YV SOD-KPDRPDELVR (EAGGNU YV SOD-KPDRPDELR (EAGGNU YV SOD-KPDRPDELLR (EAGGNU YV) SOD-KPDRPDELLR (EAGGNU YV) SOD-KPDRPDELLR (EAGGNU YV) SOD-KPDRPDELLR (EAGGNU YV) SOD-KPDRPDELLR (EAGGNU YV)	W T M  CGRVLGVLANSRAILCDNYLKPYVI S  CGRVLGVLANSRAILCDNYLKPYVI S  CGRVLGVLANSRAILCDNYLKPYVS  CGRVLGVLANSRAILCDNYLKPVI S  CGRVLANSRAILCDNYLKPVI S  CGRVLANSRAILCDNYLKPVI S	327 309 275 306 287 297 304 329 304 329 304 306 310 329 326 304 406 362 399 424 400 391 399 424 400 391 399 424 400 391 399 424 400 391 399 424 400 301 309 300 301 309 300 300 300 300 300 300 300 300 300
DEEVC.         EGNLKACSSN.         GRELOTE           VERVAMCRECCI CVCN.         GRELOTE           VKEVAMCRECCI CVCN.         GRELOTE           VKEVSCRECLIVNCATRSIKKSGRELCSFC         GRELOTE           VKEVSCRECLIVNCATRSIKKSGRELCSFC         GRELOTE           VKEVSCRESN         GRELOTE           DEV.         RRCSNTFT.         GRELOTE           DEV.         RRSVESAN.         GRELOTE           DEV.         RRSVESAN.         GRELOTE           DEV.         RRSVESAN.         GRELOTE           DEV.         RRSVESAN.         GRELOTE           DEV.         REGECOSH.         GRELOTE           DEV.         LENEVISAN.         GRELOTE           DEV.         LENEVISAN.         GRELOTE           DEV.         LENEVISAN.         GRELOTE           DEV.         LENEVISAN.         GRELOTE           DEV.         LANCHART, GRELOTE         GRELOTE           DEV.         LANCHART, GRELOTE         GRELOTE           DEV.         LANCUACSPN.         GRELOTE           DEV.         LANCUACSPN.         GRELOTE           DEV.         LANCUACSPN.         GRELOTE           DEV.         LANCUACSPN.         GRELOTE	9 SVC9777 MEX VV7PEM CDAVGSTAVAVV7PEM SEAACSTAVASV17PEM USX05774X5017PCM CDAVGSTAVS017PCM CDAVGSTAVS017PCM CDAVGSTAVS017PCM SDAVGSTAVS017PCM SDAVGSTAVS017PCM SDAVGSTAVAV7PCM SDAVGSTAV	P R SACCEDSRAULCR GAAVE ACCEDSRAULCR GAAVE ACCEDSRAULCR GAAVE SACCEDSRAULCR GAAVE SACCEDSRAULCR GAAVE SACCEDSRAULCR GAAVE ACCEDSRAULCR GAAVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACCEDSRAUVE ACC	V GGNGCANVA C V SOLKKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDEL R GAAGGRVI YV SOLKPDRPDEL R GAAGGRVI YV SOLKPDRPDEL R GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDEL R GAAGGRVI YV SOLKPDRPDRVI YV SOLKPDRPDRVI YV SOLKPDRPDRVI YV SOLKPDRVV YV SOLVC YV SOLKPDRVV YV SOLKPDRVV YV SOLVC YV	W T M CGRAVL GULANSRAI CDNYL KPYVI S CGRAVL GULANSRAI CDNYL KPYVI S CGRAVL GULANSRAI CDNY KPYVI S CGRAVL GULANSRAI CDNYL KPYVI S CGRAVL S CGRAVL S CGRAVL S CGRAVL S CGRAVL S CGRAVL S CGRAVL S CGRAVL S CGRAVL S	327 309 275 306 287 297 304 329 304 329 304 310 299 326 304 423 304 423 362 399 381 400 362 399 381 400 423 399 424 399 424 432 426
DEEVC.       EGNLKACSSN.       GR GELGTE         VKEVANCRECCICVCN.       GR GELGTE         VKEVANCRECCICVCN.       GR GELGTE         VKEVSCRECLVNGATRSKKSGR GR GELGSE       GR GELGTE         DEV.       SKELGTE       GR GELGTE         DEV.       LESUVSAN       GR GELGTE         DEV.       LEGGCVS-       GR GELGTE         DEV.	9 SVC9777 MEX VANTPER CDAVGSTAV GAVATPER SEACSTAV GAVATPER CDAVGSTAV GAVATPER COMPACIE GAVGTAV GAVATPER COMPACIE GAVGTAV GAVATPER COMPACIE	P R SACCEDSRAULCR CAAP F ACCEDSRAULCR CAAP F AAP A ACCEDSRAULCR CAAP F AAP A ACCEDSRAULCR CAAP F ACCEDSRAULCR CAAP F AAP A ACCEDSRAULCR CAAP F AAP A ACCEDSRAUCR CAAP F	V d9h9cshva c v SOLKKPDRPDELVR GACGGV V SOLKKPDRPDELVR GACGGV V SOLKPDRPDELVR GACGGV V SOLKPDRPDELLR GACGGV V SOLKPDRPDELLR GACGGV V SOLKPDRPDELLR GACGGV V SOLKPDRPDELLR GACGGV V SOLKPDRPDELLR GACGGV V SOLKPDRPDELLR GACGV V SOLVOV V SOLVARDPOV V SOLVOV V SOLVARDPV V SOLVARDPV V SOLVARDPV V SOLVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVVV	W T M CGRVLGVLANSRAICDNVLKFYVI S CGRVLGVLANSRAICDNVLKFYVI S CGRVLGVLANSRAICDNVLKFYVS CGRVLGVLANSRAICDNVLKFYS	327 309 275 306 287 297 304 329 304 329 304 300 299 326 304 423 304 423 304 423 304 423 304 423 309 381 400 399 381 400 399 424 366 402 424 400 402 424 400 402 424 400 402 424 400 402 424 400 424 329 424 400 424 424 400 424 424 424 424 424
DEEVC.         EGNLKACSSN.         GR GELGTE           VREVAWCRECGI CVCN.         GR GELGTE           VREV.         SWG. DSVTAN.         GR GELGTE           VREV.         SWG. ESVVSAN.         GR GELGTE           DEEV.         RRSCSNTFT.         GR GELGTE           DECV.         REGOCVSH.         GR GELGTE           DECV.         LENLVACEFN.         GR GELGTE           DECV.         LSSSCTVT.         GR GELGTE           DECV.         SMSSSCTVT.         GR GELGTE           GEV.         SMSSSCTVT.         GR GELGTE           DEV.         LANDERCULACEFN.         GR GELGTE           GEV.         LEGELLACEFN.         GR GELGTE           GEV.         LEGELACLACEFN.         GR GELGTE           GEV.         LEGELACLACEFN.         GR GELGTE </td <td>9 SVC9777 MEX V V PER V CDAVGETA V AVV PER V CDAVGETA V AVV PER V CDAVGETA V SVV PER V CDAVGETA V SVV PER V CDAVGETA V SVV PER V DAVGETA V SVV PER V SAVGETA V AVV PER V DAVGETA V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER</td> <td>P NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH SNCCCSRAULCRGAPH SNCCCSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCSSRAULCRGAPH NCCSSRAULCRGAPH NCCCSSRAULCRGAPH NCCSSSS NCCSSRAULCRGAPH NCCSSSS NCS</td> <td>V G9h9Cshva C V SOLKFDRPDELVR EANGGNU YV SUD-KFDRPDELVR EANGGNU YV SUD-KFDRPDELVR EANGGNU YV SUD-KFDRPDEL R EANGGNU YV SUD-KFDRPDEN R EANGGNU YV SUD-K</td> <td>W 1 M CGRIVL GULANSRAI CENNUK KPYVI S CGRIVL GULANSRAI CENNUK KP</td> <td>327 309 275 306 287 297 324 329 324 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 320 320 320 320 320 320 320 320 320 320</td>	9 SVC9777 MEX V V PER V CDAVGETA V AVV PER V CDAVGETA V AVV PER V CDAVGETA V SVV PER V CDAVGETA V SVV PER V CDAVGETA V SVV PER V DAVGETA V SVV PER V SAVGETA V AVV PER V DAVGETA V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER V SVV PER	P NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH SNCCCSRAULCRGAPH SNCCCSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCDSRAULCRGAPH NCCCSSRAULCRGAPH NCCSSRAULCRGAPH NCCCSSRAULCRGAPH NCCSSSS NCCSSRAULCRGAPH NCCSSSS NCS	V G9h9Cshva C V SOLKFDRPDELVR EANGGNU YV SUD-KFDRPDELVR EANGGNU YV SUD-KFDRPDELVR EANGGNU YV SUD-KFDRPDEL R EANGGNU YV SUD-KFDRPDEN R EANGGNU YV SUD-K	W 1 M CGRIVL GULANSRAI CENNUK KPYVI S CGRIVL GULANSRAI CENNUK KP	327 309 275 306 287 297 324 329 324 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 310 329 326 320 320 320 320 320 320 320 320 320 320
DEEVC. EGNLKACSSN	9 SVC9777 MEX VVTPEN SEACCTAV SVTPEN SEACCTAV SVTPEN UDAVCSTAV SVTPEN UDAVCSTAV SVTPEN UDAVCSTAV SVTPEN SVTPEN SVTV SVTV SVTV SVTV SVTV SVTV SVTV SVT	P R Records and constructions	V G9h9Cshva C V SOLKKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELVR GAAGGRVI YV SOLKPDRPDELIR GAAGGRVI YV SOLKPDRPDEV SV VV DAAGGRVI YV SOLKPDRPDEV SV VV DAAGGRVI YV SOLVARDRPDEV SV VV DAAGGRVI VV SOLVARDRPDEV SV VV DAAGGRVI VV SOLVARDRPDEV SV VV DAAGGRVI VV DAAGVVV DAAGVVVV DAAGVVVVVVVVVVVVVVV	W T TO CERVE GULANSRAI CENVE KEYVES CERVE KEYVES CERVES CERVE KEYVES CERVES CERVES CERVES CERVES CERVES CERVES CERVES CERVES	327 309 275 306 287 297 304 329 304 306 310 329 326 304 423 304 423 304 423 304 423 304 423 304 423 309 326 309 326 400 411 399 424 400 411
DEEV.C. EGNLKACSSN	9 SVC9777 MEX V TPEM CDAVGSTA AAVTPEM EDS/GSTA VSVTPEM CDS/GSTA VSVTPEM CDS/GSTA VSVTPEM CDS/GSTA VSVTPEM CDS/GSTA VSVTPEM CDAVGSTA VSVTPEM DAVGSTA VSVTPEM DA	P A SNCCDSRAULCREANT NCCDSRAULCREANT	V G9h9CshV3 C V SOL+KPDRPDELV, GEAGNU YV SOL+KPDRPDELV, GEAGNU YV SOL+KPDRPDELV, GEAGNU YV SOL+KPDRPDELL, GEAGGNU YV SOL+KPDRPDELL, GEAGNU YV SOL+KRPDRPDELL, GEAGNU YV SOL+KPDRPDELL, GEAGNU YV SOL+KPDRPDEL, GEAGNU YV	W T M CGRVLGULANSRAILCDNYLKFYVI S CGRVLGULANSRAILCDNYLKFYVI S CGRVLGULA	327 309 275 306 287 297 324 329 324 304 306 310 304 306 310 309 326 304 423 406 362 304 423 400 399 381 400 399 381 400 399 424 436 402 426 401 402 426 402 426 402 426 402 426 402 426 402 426 402 426 402 426 402 426 402 426 402 426 402 426 402 402 402 402 402 402 402 402 402 402
DEEVC.         EGNLKACSSN.         GRELOTE           VREVAWCRECCI CVCN.         GRELOTE           VREVAWCRECCI CVCN.         GRELOTE           VREVSCRECL VNGATRSIKKSGRELOSE         GRELOTE           VREVSCRECL VNGATRSIKKSGRELOSE         GRELOTE           VREVSCRASS         GRELOTE           VREVSKAS         GRELOTE           DEV.         RREGEVEN           DEV.         RRESVESAS           VREVSKAS         GRELOTE           DEV.         REGEVEN           DEV.         REGEVEN           DEV.         REGEVEN           DEV.         REGEVEN           DEV.         IVSKANSSA           DEV.         IVSKANSA           DEV.	9 SVC9777 MEX V V PER CDAVGSTAVAVYPER SEACSTAVASVI PEC LDAVGSTAVSVI PEC LDAVGSTAVSVI PEC LDAVGSTAVSVI PEC LDAVGSTAVSVI PEC SOLVETAVSVI PEC LDAVGSTAVSVI PEC SDAVGSTAVSVI PEC LDAVGSTAVSVI PEC SDAVGSTAVSVI PEC LDAVGSTAVSVI PEC SDAVGSTAVSVI PEC LDAVGSTAVSVI PEC LDAVGSTAVSVI PEC SDAVGSTAVSVI PEC SDAVGSTAV	P RESERVICE GAN F NCCEDSRAULCR GAN F NCCEDSRAULCR GAN F NCCEDSRAULCR GAN F SNCEDSRAULCR GAN F SNCEDSRAULCR GAN F NCCEDSRAULCR GAN F NCCEDSRAU CR GAN F NCCEDSR	V G9h9Cshva C V SOLKKPDRPDELVR EANGGNU YV SOLKPDRPDELVR EANGGNU YV SOLKPDRPDELVR EANGGNU YV SOLKPDRPDEL R EANGGNU YV SOLKPDRPDRPDU R EANGGNU YV SOLKPDRPDU R EANGGNU YV SOLV R EANGGNU R EANGGNU YV SOLV R EANGGNU R	W         I           CGRAVL GULANSRAI CDNYLKPYVI S         CGRAVL GULANSRAI CDNYLKPYVI S           CGRAVL GULANSRAI CDNYLKPYVI S <t< td=""><td>327 309 275 306 327 297 297 304 329 304 329 304 300 299 326 304 423 304 362 399 381 399 381 399 391 399 326 304 400 391 399 326 304</td></t<>	327 309 275 306 327 297 297 304 329 304 329 304 300 299 326 304 423 304 362 399 381 399 381 399 391 399 326 304 400 391 399 326 304
	9 8VC9777 MEX VANTPEN 9 8VC9777 MEX VANTPEN 98AACSTAV SAVI AFDO 105/05174/105/11FDO 101/05/05174/105/11FDO 101/05/05174/105/11FDO 101/05/05174/105/11FDO 101/05174/105/1150/105 101/05174/105/1150/105 101/05174/105/1150/105 101/05174/105/1150/105 101/05174/105/1150/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105/105 101/05174/105 101/05174/105 10	P R SACCEDSRAULCR GAAP ACCEDSRAULCR GAAP ACCEDSRAUCR GAAP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP ACCEDSRAUP	V G9h9Cshva C V SOL-K-PDR-PDEL VR EAAGGRVI YV SOL-K-PDR-PDEL VR EAAGGRVI YV SOL-K-RANGRVI VR EAAGGR	w         1           CGRAVLGULANSRAILCDNULKFYVI S         CGRAVLGULANSRAILCDNULKFYVI S	327 309 275 304 327 269 304 329 304 329 304 310 329 304 360 310 304 360 362 304 362 304 362 304 362 304 362 304 362 304 362 304 362 304 304 362 304 304 304 304 304 304 304 304 304 304

**Figure 1.** The amino acid sequence analysis of MdPP2C24/37. (**A**) Both MdPP2C24/37 were predicted to have PP2C catalytic domains. (**B**) Sequence analysis indicated that MdPP2C24/37 are highly conserved proteins analogous to proteins in *Arabidopsis thaliana*, *Arabidopsis lyrata* subsp. *Lyrata*, *Arachis ipaensis*, *Brassica napus*, *Cicer arietinum*, *Eutrema salsugineum*, *Glycine max*, *Rosa chinensis*, *Prunus persica*, *Prunus avium*, and *Prunus dulcis*.

# 2.2. MdPP2C24/37 Genes Are Induced by ABA Treatment or Mannitol Stress in Apple

To identify the cellular localization of MdPP2C24 and MdPP2C37, we performed in vivo transient expression analysis in tobacco protoplasts. *355*::MdPP2C24-GFP, *355*::MdPP2C37-GFP, and *355*::GFP constructs were transformed into tobacco leaf protoplasts using a polyethylene glycol (PEG)-mediated method [29]. The fluorescence sig-



nal of MdPP2C24/37-GFP fusion proteins was observed in the nucleus, indicating that MdPP2C24/37 are nuclear proteins (Figure 2A).

**Figure 2.** Nucleus-located genes *MdPP2C24/37* were induced by ABA or mannitol. (**A**) Subcellular localization of MdPP2C24/37-GFP fusion proteins in tobacco protoplasts. *35S*::GFP alone or MdPP2C24/37-GFP correspond to chlorophyll and bright field images, respectively, and the superposition of fluorescent illumination, chlorophyll, and bright field images is shown. (**B**) Real-time qPCR analysis of *MdPP2C24/37* expression in apple leaves. Total RNA was isolated from the ABA- or mannitol-treated apple leaves and used for real-time qPCR. Results represent mean  $\pm$  SE from three independent experiments, with similar results obtained. Values were significantly different from WT at \* *p* < 0.05, \*\* *p* < 0.01 or \*\*\* *p* < 0.001.

To preliminarily study the MdPP2C24/37 response to drought stress in apple, we performed a 50  $\mu$ M ABA or 200 mM mannitol treatment on apple leaves (Figure 2B). The transcriptional analysis results using qRT-PCR supported the possibility that MdPP2C24/37 are involved in ABA or osmotic signaling in apple.

# 2.3. MdPP2C24/37 Regulate ABA Response in Germination and Seedling Establishment through Stress-Responsive Genes in Arabidopsis

To study the gene function of *MdPP2C24/37* properly, we generated *MdPP2C24/37* overexpression lines in Arabidopsis driven by the cauliflower mosaic virus (CaMV) 35S promoter. PCR analysis of DNA levels and real-time qPCR analysis of RNA transcript levels confirmed *MdPP2C24/37* overexpression in these lines (Supplementary Figure S2). Three independent overexpression lines were obtained for each MdPP2C. Then, overexpression lines MdPP2C24 (24-2, 24-6) and MdPP2C37 (37-4, 37-7) were chosen as representative lines. To study the roles of MdPP2Cs in the ABA response, we germinated the plants in MS media containing different concentrations of ABA. After treatment with 0.5 or 1 µM ABA, the seed germination rates of the MdPP2C24/37 overexpression lines were significantly higher than those of WT (Figure 3A,B). In addition, the MdPP2C24/37 overexpression lines exhibited markedly higher cotyledon greening rates under the 0.5  $\mu$ M ABA treatment than WT, including over 50% in the *MdPP2C24* overexpression lines and over 20% in the *MdPP2C37* overexpression lines. In contrast, only a 4% cotyledon greening rate was recorded in the wild type plants. The same cotyledon greening rate trend was observed after treatment with 1  $\mu$ M ABA; namely almost 40% in the *MdPP2C24* overexpression lines and 2% in the MdPP2C37 overexpression lines, whereas no cotyledon greening was observed in WT (Figure 3C). In these results, no significant difference was observed among plants under control conditions (Figure 3A–C, Supplemental Figure S3). Next, the 7-day-old seedlings were treated with liquid MS medium to which 50 µM ABA was added. A qPCR assay was used to detect the stress-responsive gene levels of DREB2A, KIN2, ABF4, and RD29A. The results showed that transcript levels of *DREB2A* and *KIN2* were notably increased in the MdPP2C24/37 overexpression lines compared to WT under control conditions. After exogenous ABA treatment, transcript levels of DREB2A and KIN2 were almost unchanged in the *MdPP2C24/37* overexpression lines; however, they were significantly increased in WT. Moreover, transcript levels of ABF4 and RD29A were upregulated after ABA treatment in all plants (Figure 3D). These results indicated that MdPP2C24/37 decreased ABA sensitivity at the germination and seedling stages in Arabidopsis.

### 2.4. Overexpression of MdPP2C24/37 Modulate Drought Tolerance

Next, the relationship between *MdPP2C24/37* and ABA-dependent drought stress response was studied. A stomatal aperture assay revealed that the *MdPP2C24/37* overexpression lines were insensitive to ABA compared with WT (Figure 4A,B). Under normal conditions, however, no differences were observed among these plants. However, higher water loss rates were observed in the detached leaves of the *MdPP2C24/37* overexpression lines at the indicated time points (Figure 4D). Furthermore, when exposed to drought stress for 15 days, fewer *MdPP2C24/37* overexpression lines survived than WT (Figure 4C).



**Figure 3.** Phenotypic analysis of overexpression of *MdPP2C24/37* in *Arabidopsis* revealed a hyposensitive phenotype to ABA at the seed germination stage. (**A**) Representative images of seed germination. (**B**) Statistical analysis of seed germination rate. (**C**) Cotyledon greening rate in WT and transgenic lines 6 days after seeds were sown in MS medium supplemented with 0, 0.5, and 1  $\mu$ M ABA. Results represent the mean  $\pm$  SD from 3 independent experiments. (**D**) Real-time qPCR analysis of stress-responsive gene expression changes in *MdPP2C24/37* overexpression lines in *Arabidopsis*. The expression levels were based on total RNA extracted from WT and *MdPP2C24/37* overexpression lines in liquid MS medium, or liquid MS medium supplemented with 50  $\mu$ M ABA for 3 h. Results represent the mean  $\pm$  SE from 3 independent experiments. The expression levels are presented as relative units, with levels under control conditions taken as 1. All experiments were replicated three times with similar results. Values were significantly different from WT at \* *p* < 0.05, \*\* *p* < 0.01 or \*\*\* *p* < 0.001.

А

С

Drought

Rewater for 3 days

Ε

Chlorophyll (mg cm<sup>-2</sup>)

0.4

0.2

0

WT

24-2 24-6 37-4 37-7

ABA





WT 24-2 24-6 37-4 37-7

Figure 4. The MdPP2C24/37 overexpression lines in Arabidopsis displayed decreased drought stress tolerance compared to WT. (A,B) Stomatal aperture assay induced by ABA of MdPP2C24/37 overexpression lines in Arabidopsis. (A) Representative images of stomatal aperture and (B) statistical analysis of stomatal aperture width/length. Values represent the mean  $\pm$  SD from three independent experiments; n = 80 per experiment. (C) Drought tolerance assay of WT and MdPP2C24/37 overexpression lines. Three-week-old plants were exposed to drought stress for 15 days and then rewatered for three days. Values represent the mean  $\pm$  SD from three independent experiments; n = 48 per experiment. (D) Water loss rates during 3 h period in detached leaves of WT and MdPP2C24/37 overexpression lines. Values represent the mean  $\pm$  SD of five individual plants per genotype. (E–G) Plants of all genotypes subjected to drought through withholding of water for 12 days. (E) Chlorophyll (F) MDA, and (G)  $H_2O_2$  levels were measured. Results represent the mean  $\pm$  SD from three independent experiments, with similar results obtained. Values were significantly different from WT at \* *p* < 0.05, \*\* *p* < 0.01 or \*\*\* *p* < 0.001.

WT 24-2 24-6 37-4 37-7

In this study, no significant differences were observed for chlorophyll, MDA, and  $H_2O_2$  levels among plants under control conditions. However, under drought stress, chlorophyll levels decreased more rapidly in all *MdPP2C24/37* overexpression lines than in WT (Figure 4E). Further, MDA and  $H_2O_2$  accumulation was significantly higher in *MdPP2C24/37* overexpression lines than in WT (Figure 4F,G). The drought stress method used in this study involved withholding water for 12 days. In general, the results presented above indicated that the *MdPP2C24/37* overexpression lines showed decreased resistance ability to ABA-mediated drought stress in adult *Arabidopsis*.

# 2.5. MdPP2C24/37 Involved in Response to Osmotic Stress through Stress-Responsive Genes in Arabidopsis

Next, the *MdPP2C24/37* overexpression lines were studied in response to osmotic stress. The germination and cotyledon greening rates of the MdPP2C24/37 overexpression lines were higher than those of WT under 200 or 300 mM mannitol treatment (Figure 5A,B). All of the MdPP2C24/37 overexpression lines displayed over 80% cotyledon greening rates, while the wild type plants only showed 35% under the 200 mM mannitol treatment. Further, the MdPP2C24 overexpression lines displayed almost 70% cotyledon greening rates, the *MdPP2C37* overexpression lines displayed 40%, and WT only displayed 7% in MS medium supplemented with 300 mM mannitol (Figure 5C). In these results, no significant difference was observed among plants under control conditions (Figure 5A–C, Supplementary Figure S3). Subsequently, the 7-day-old seedlings were treated with liquid MS medium containing 200  $\mu$ M mannitol. A real-time qPCR assay was used to detect the stress-responsive gene levels of DREB2A, KIN2, ABF4, and RD29A. DREB2A and KIN2 expression levels in the MdPP2C24/37 overexpression lines were much higher than those in WT under control conditions. Under exogenous mannitol treatment, transcript levels of DREB2A and KIN2 were almost unchanged in the MdPP2C24/37 overexpression lines; however, most transcript levels were significantly increased in WT. Further, transcript levels of *ABF4* and *RD29A* were upregulated after mannitol treatment in all plants (Figure 5D). To summarize, MdPP2C24/37 decreased osmotic sensitivity at the germination and seedling stages in Arabidopsis.

# 2.6. Both MdPP2C24/37 Interacted with MdPYL2/12 In Vitro and Vivo

In this study, the coding sequences of MdPP2C24/37 were used as prey and several possible MdPYLs/PYR1 were used as bait in a yeast two-hybrid assay. Under the four-minus selection medium (SD/-Leu/-Trp/-His/-Ade) growth conditions, MdPYR1 was unable to interact with MdPP2Cs, while MdPYL2/9/12 interacted with MdPP2C24/37 in vitro (Figure 6A).

To further confirm the associations of MdPP2C24/37 and MdPYLs/PYR1 in vivo, we performed a BiFC assay. Fluorescence signals were observed only when MdPP2C24/37 and MdPYL2/12 interacted (Figure 6B). MdPP2C24/37 and MdPYL2/12 interacted in the nucleus, which was stained with the nucleic acid dye DAPI. The interaction between YFP<sup>C</sup>-AtCARK3 + YFP<sup>N</sup>-AtPYL1 was adopted as a positive control, while interactions between YFP<sup>C</sup>-MdPP2C24/37 + YFP<sup>N</sup> and YFP<sup>C</sup> + YFP<sup>N</sup>-MdPYL2/12 were adopted as negative controls (Supplementary Figure S4A). However, no fluorescence signal was detected between MdPP2C24/37 and MdPYL9/PYR1 (Supplementary Figure S4B). These results indicated that both MdPP2C24/37 interacted with MdPYL2/12 in vitro and in vivo.



**Figure 5.** Phenotypic analysis of *MdPP2C24/37* overexpression lines in *Arabidopsis*, which caused a hyposensitive phenotype to mannitol at the seed germination stage. (**A**) Representative images of seed germination. (**B**) Statistical analysis of seed germination rate. (**C**) Cotyledon greening rates of WT and transgenic lines 6 days after seeds were sown in MS medium supplemented with 0, 200, and 300 mM mannitol. Results represent the mean  $\pm$  SD from 3 independent experiments. (**D**) Real-time qPCR analysis of stress-responsive gene expression changes in the *MdPP2C24/37* overexpression lines in *Arabidopsis*. The expression levels were based on total RNA extracted from WT and transgenic *Arabidopsis* in liquid MS medium, or liquid MS medium supplemented with 200 Mm mannitol for 3 h. The expression levels are presented as relative units, with levels under control conditions taken as 1. Results represent the mean  $\pm$  SE from 3 independent experiments, with similar results obtained. Values were significantly different from WT at \* *p* < 0.05, \*\* *p* < 0.01 or \*\*\* *p* < 0.001.



**Figure 6.** MdPP2C24/37 interacted with MdPYL2/12 in vitro and vivo. (**A**) MdPP2C24/37 interacted with MdPYL2/9/12 in the yeast two-hybrid assay. AD–MdPP2C24/37 fusion prey vectors were cotransformed with BD–MdPYL2/9/12/PYR1 fusion bait vectors into yeast cells. Positive interactions were indicated by the ability of cells to grow on SD/–Leu/–Trp/–His/–Ade dropout medium. Empty AD prey vector and BD bait vectors were used as negative controls. (**B**) MdPP2C24/37 interacted with MdPYL2/12 in the bimolecular fluorescence complementation (BiFC) assay, showing fluorescence in nuclei of tobacco leaf epidermal cells. The C-terminus part of YFP was fused to MdPP2C24/37, and the N-terminus part of YFP was fused to MdPYL2/12. All experiments were replicated three times, with the same results obtained.

# 3. Discussion

Adverse environmental cues severely affect plant growth and development. To deal with stress, plants have developed a series of strategies, such as ABA signal conduction [14,30–32]. PP2Cs, important signal transductors in ABA signaling, have been reported in species such as *Arabidopsis* [33,34], maize [24], soybean [35], *Pyrus bretschneideri* [26], *Brassica rapa* [36], wheat [37], and *Gossypium hirsutum* [38]. However, the PP2C gene family has not been studied widely in the *Rosaceae* family, represented by apple. In this study, MdPP2C24/37, having highly conserved PP2C catalytic domains, were identified through sequence alignment (Figure 1, Supplementary Figure S1) according to bioinformatics analysis [26], a method which is consistent with previous studies [39].

Considerable evidence indicates that PP2C localization is required for different functions. In *Arabidopsis*, plasma membrane-localized members of PP2Cs, namely PP2C.D2, PP2C.D5, and PP2C.D6, are major regulators of cell expansion through their physical interaction with SAUR19 and PM H<sup>+</sup>-ATPases, and they inhibit cell expansion by dephosphorylating the penultimate threonine of PM H<sup>+</sup>-ATPases [40]. AtABI1, a nuclear protein, is essential to confer insensitivity towards ABA [41]. In this study, MdPP2C24/37 were nucleus-located and induced by ABA (Figure 2). The results indicated that both MdPP2Cs are probably involved in nuclear signal transduction, which prompted us to analyze their functions in ABA responsiveness. Further results revealed that the expression of *MdPP2C24/37* in *Arabidopsis* led to plant insensitivity to ABA during seed germination and seedling establishment and to ABA-induced drought stress (Figures 3 and 4, Supplementary Figure S3). Previous reports revealed that PP2Cs are involved in ABA signaling through their interaction with ABA receptors PYR/PYLs [32,42–45]. However, whether MdPP2Cs associate with MdPYLs/PYR1 in apple is not clear. In this study, MdPP2C24/37 were found to interact with ABA receptors MdPYL2/12 in the nucleus through a yeast two-hybrid assay and BiFC assay (Figure 6). The results proved that MdPP2C24/37 are negative regulators, as typical PP2Cs, in ABA signaling. However, MdPYL9, a homologue to PYL9 in *Arabidopsis, Zea mays*, and *M. domestica* [46–48], was not observed to interact with MdPP2C24/37 in vivo (Figure 6, Supplementary Figure S4). In summary, these results suggest that MdPP2C24/37 interact with MdPYL2/12, specifically in the nucleus, and play negative roles in ABA signaling.

In higher plants, ABA signaling can be triggered by multiple stresses, such as drought and osmotic stress [27]. In addition, some PP2Cs play pivotal roles in osmotic stress [49]. For instance, *ZmPP2C-As* in maize has been found to be dramatically induced by osmotic stress, resulting in a higher seed germination rate under osmotic stress and higher death rate when plants are exposed to drought stress [24]. OsPP108 and OsSIPP2C1 in rice, which are upregulated by high salt, exogenous ABA, and drought treatment, act as negative regulators in ABA signaling [50,51]. In this study, mannitol was used to reveal the roles played by MdPP2C24/37 in the relationship between ABA and osmotic stress signaling. The results indicated that transcript levels of MdPP2C24/37 were induced by ABA or mannitol (Figure 2). The *MdPP2C24/37* overexpression lines were hyposensitive at the germination and seedling establishment stages under ABA or mannitol treatment in Arabidopsis (Figures 3 and 5, Supplementary Figure S3). Further, stress-related genes, namely DREB2A, KIN2, ABF4, and RD29A, exhibited almost the same expression tendencies under ABA or mannitol treatment in all *MdPP2C24/37* overexpression plants (Figures 3 and 5). Plants induce stomatal movement through ABA signaling [32] and closely guard cells to maintain water balance when they are exposed to drought stress [52]. In this study, all of the *MdPP2C24/37* overexpression lines displayed inhibited stomatal closure in response to exogenous ABA treatment (Figure 4). Moreover, osmotic stress, which accompanies water deficit, can be regulated through ABA-dependent signaling [28,53]. Overall, this study comprehensively reported the roles of MdPP2C24/37 at different stages. At the seed germination and seedling establishment stages, *MdPP2C24/37* overexpression plants were hyposensitive to both ABA and mannitol. At the adult stage, overexpression of MdPP2C24/37 in Arabidopsis led to a lower survival rate through its inhibition of stomatal closure (Figures 3–5). These results are consistent with those found for *PtNF-YA9* (NUCLEAR FACTOR Y9) in *Populus trichocarpa* [54]. In summary, these observations demonstrate that MdPP2C24/37 participate in multiple signaling pathways at different growth stages and function as connecting links in response to ABA signaling and osmotic stress.

Current evidence suggests that redundancies in PP2Cs create major obstacles to studying the functions of PP2Cs genes using genetic approaches [55]. For instance, ABI1 and ABI2 phosphatases play overlapping roles in controlling ABA actions [10]. It is noteworthy that both MdPP2C24/37 have highly conserved PP2C catalytic domains but share only 54% homologous similarity (Figure 1). This analysis suggests that MdPP2C24/37 may have different functions, as well as redundant functions, in ABA signaling in apple. That is, the functions of MdPP2C24/37 may diverge at some point in response to ABA and osmotic stress, and the functions of their potential targets may be different from those of MdPYL2/12 (Figure 6). Therefore, these synergistic effects further suggest that MdPP2C24/37 may participate in other signaling pathways.

#### 4. Materials and Methods

# 4.1. Plant Materials and Growth Conditions

Subcultured *'huashuo'* apple [3] shoots were propagated in MS medium supplemented with 2.0 mg L<sup>-1</sup> TDZ (thidiazuron, Cat. #P6186, Sigma-Aldrich, America), 0.1 mg L<sup>-1</sup> IBA (3-indole butyric acid, Sigma-Aldrich), 30 g L<sup>-1</sup> sucrose, and 7 g L<sup>-1</sup> agar (pH 5.8). Seeds

of *Arabidopsis thaliana* (WT, Col-0) and *Nicotiana benthamiana* were sterilized by soaking them in 20% NaClO (v/v) for 15 min, and they were washed 6 times to remove residual NaClO solution. Sterilized seeds were sown in MS medium containing 2% sucrose and 0.8% agar (pH 5.8), and vernalized at 4 °C for 3 days. All plants were cultivated at 22 °C under long day conditions with a cycle of 16 h light/8 h dark) and 180 µmol m<sup>-2</sup> s<sup>-1</sup>.

# 4.2. Real-Time qPCR Analysis

Total RNA of fresh leaves from four-week-old subculture apple shoots were extracted using the CTAB assay [56]. RNA of *A. thaliana* was isolated from 7-day-old seedlings using RNAiso Plus (Cat. #9109, Takara, Japan). Reverse transcription was performed using the cDNA Synthesis Kit (Cat. #RR047A, Takara, Japan). Real-Time qPCR was performed using SYBR Premix Ex Taq (Cat. # RR430A, Takara, Japan). *ACTIN2 (At3g18780)* was used as an internal control for *Arabidopsis*, while *MdEF-1a* (*LOC103443462*) was used as an internal control for apple. All gene-specific primer pairs are listed in Supplementary Table S1.

#### 4.3. Amino Acid Sequence Alignment and Phylogenetic Analysis

The predicted amino acids of MdPP2C24/37 and their homologous proteins were obtained from NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 1 January 2022) and JGI (https://phytozome.jgi.doe.gov/pz/portal.html#, accessed on 1 January 2022). The homologous proteins of MdPP2C24/37 in *A. thaliana, Arabidopsis lyrata* subsp. *Lyrata, Arachis ipaensis, Brassica napus, Cicer arietinum, Eutrema salsugineum, Glycine max, Rosa chinensis, Prunus persica, Prunus avium,* and *Prunus dulcis* were obtained using the NCBI BLAST tool. The protein domain prediction map was created using IBS1.0.2 software (http://ibs.biocuckoo.org/, accessed on 1 January 2022). The phylogenetic tree was generated using the Neighbor-Joining method in MEGA 7 software, with 500 replicated bootstrap values used at each node [57]. Sequence alignment was performed using DNAMAN9 (http://www.lynnon.com/, accessed on 1 January 2022).

### 4.4. Transient Expression Assay and Transgenic Arabidopsis Constructs

To achieve transient expression in tobacco mesophyll cell protoplasts, the MdPP2C24/37 CDSs were cloned into the *p*BI221-*eGFP* vector. Analysis of transient expression in protoplasts was performed as previously described [29].

To generate transgenic *Arabidopsis*, MdPP2C24/37 CDSs were cloned into the *p*Cambia2300 vector. The plasmids were introduced into *Arabidopsis* Col-0 by *Agrobacterium tumefaciens* (GV3101)-mediated transformation. The floral dip method was adopted in this study [58]. The seeds of the transgenic plants were screened in MS medium supplemented with 35 mg/L kanamycin. The T3 homozygous seeds were used for phenotypic analysis. The primers used in this assay are listed in Supplementary Table S1.

### 4.5. Phenotype Analysis

For germination and seedling establishment assays, approximately 150 sterilized seeds of each line were sown in MS medium with various concentrations of ABA (Cat. #90769, Sigma-Aldrich, America) or mannitol (Cat. #M8140, Solarbio, China). The germination rate was calculated every 12 h 6 days after sowing. The cotyledon greening rates were calculated on the 6th day after sowing.

For the seedling establishment assay, seeds grew vertically in 1/2 MS medium for 7 days with 0 or 0.5  $\mu$ M ABA or 300 mM mannitol. The root lengths were measured using ImageJ software (https://imagej.en.softonic.com/, accessed on 1 January 2020).

For the stomatal aperture assay, rosette leaves of 3-week-old plants were detached and floated in stomatal opening solution (5 mM KCl, 10 mM MES (2- (N-morpholino) ethanesulfonic acid), 50  $\mu$ M CaCl2, pH 6.15) in the dark for 2 h, then exposed to continuous light for 2 h, followed by the addition of 10  $\mu$ M ( $\pm$ ) ABA. In order to estimate ABAinduced stomatal closure, apertures were recorded on epidermal strips after 2 h incubation. Width/length ratios were measured and counted using ImageJ software (v1.8.0, National Institutes of Health, America).

For the water loss assay, rosette leaves of 3-week-old *Arabidopsis* plants were detached. The water loss rate was monitored at indicated times. For drought stress analysis, 3-week-old plants were not watered for 15 days and then rewatered. The survival rate was calculated.

#### 4.6. Determination of Drought-Responsive Physiological Indices

The 3-week-old *Arabidopsis* plants were subjected to normal conditions or exposure to drought stress. Then, leaves were collected for chlorophyll, malondialdehyde (MDA), and  $H_2O_2$  content determination according to a previously described protocol [59].

### 4.7. Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed according to a previously described protocol [60]. *MdPYL2 (LOC103440595, MDP0000147358), MdPYL9 (LOC103413926, MDP0000284624), MdPYL12 (LOC103434883, MDP0000132875),* and *MdPYR1 (LOC103436894, MDP0000125850)* were investigated in this study. The CDSs of MdPP2C24/37 were cloned into the pGADT7 vector (AD). The CDSs of MdPYL2, MdPYL9, PYR1, and MdPYL12 were cloned into the pGBKT7 vector (BD).

The AD-MdPP2C24/37 and BD-MdPYL2/9/12/PYR1 plasmids were co-transformed into yeast AH109 cells. The yeast cells were plated in SD/-Leu/-Trp medium for 2–3 days, and in SD/-Leu/-Trp/-His/-Ade medium for 5–8 days. AD-AtCARK3+BD-AtPYL1 was used as the positive control [61].

### 4.8. Bimolecular Fluorescence Complementation (BiFC) Assay

The BiFC assay was performed according to a previously described protocol [62]. The BiFC vectors pSPYNE (YFP<sup>N</sup>)/pSPYCE (YFP<sup>C</sup>) harbor either the N-terminus or C-terminus of eYFP. YFP<sup>N</sup> was fused to the C termini of MdPYL2/9/12, and PYR1 and YFP<sup>C</sup> were fused to the C termini of MdPP2C24/37. The vectors were transformed into *A. tumefaciens* GV3101. Pairwise construct combinations were transiently expressed in *N. benthamiana* epidermal cells. The previously described YFPC-AtCARK3 and YFPN-AtPYL1 constructs were used as positive controls [34]. The fluorescence signal of YFP was detected after infiltration for 3 days using an Olympus optical microscope at an excitation wavelength of 560 nm. The primers used for these constructs are listed in Supplementary Table S1.

#### 4.9. Statistical Analysis

Data are represented as the mean  $\pm$  SD and  $\pm$  SEM. Statistical analysis was performed using Student's *t*-test. Values were significantly different from WT at \* *p* < 0.05, \*\* *p* < 0.01 or \*\*\* *p* < 0.001.

#### 5. Conclusions

In conclusion, this study demonstrated that MdPP2C24/37, isolated from '*huashuo*', are nucleus-located and negatively regulate seed germination, seedling establishment, stomatal aperture, and stress-related gene expression in response to drought stress. The interactions between MdPP2C24/37 and ABA receptors MdPYL2/12 provide new a direction for studying the ABA transduction mechanism in apple. Overall, these results provide evidence that *MdPP2C2C24/37* respond to ABA and osmotic stress, thus enriching the ABA signal regulation network in apple.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232214375/s1.

**Author Contributions:** Y.-Y.L., Y.Y. and X.-Y.L. conceived and designed the research. Y.-Y.L., Y.L., W.-S.S., X.-M.G., B.H. and H.-R.S. performed the experiments. W.-S.S. analyzed the data. Y.-Y.L., Y.Y., X.-F.L., Z.-B.L. and J.-M.W. finished writing the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** Supported by the Open Project Program of Panxi Crops Research and Utilization Key Laboratory of Sichuan Province (No. SZKF2105) and the National Science Foundation of China (NSFC grants 31870240).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

# References

- 1. Naor, A.; Naschitz, S.; Peres, M.; Gal, Y. Responses of apple fruit size to tree water status and crop load. *Tree Physiol.* **2008**, *28*, 1255–1261. [CrossRef] [PubMed]
- Wang, Y.; Liu, L.; Wang, Y.; Tao, H.; Fan, J.; Zhao, Z.; Guo, Y. Effects of soil water stress on fruit yield, quality and their relationship with sugar metabolism in 'gala' apple. *Sci. Hortic.* 2019, 258, 108753. [CrossRef]
- 3. Yan, Z.; Zhang, H.; Guo, G.; Zhang, S.; Liu, Z. Selection of a new early-ripening apple cultivar-huashuo. *J. Fruit Sci.* 2010, 27, 655–656.
- 4. Velasco, R.; Zharkikh, A.; Affourtit, J.; Dhingra, A.; Cestaro, A.; Kalyanaraman, A.; Fontana, P.; Bhatnagar, S.K.; Troggio, M.; Pruss, D.; et al. The genome of the domesticated apple (*Malus* × *Domestica borkh*.). *Nat. Genet.* **2010**, *42*, 833–839. [CrossRef]
- Song, J.; Yang, F.; Xun, M.; Xu, L.; Tian, X.; Zhang, W.; Yang, H. Genome-wide identification and characterization of vacuolar processing enzyme gene family and diverse expression under stress in apple (*Malus × Domestic*). Front. Plant Sci. 2020, 11, 626. [CrossRef] [PubMed]
- 6. Zhu, J.K. Abiotic stress signaling and responses in plants. *Cell* **2016**, *167*, 313–324. [CrossRef] [PubMed]
- Nakashima, K.; Yamaguchi-Shinozaki, K. Aba signaling in stress-response and seed development. *Plant Cell Rep.* 2013, 32, 959–970. [CrossRef]
- 8. Leung, J.; Giraudat, J. Abscisic acid signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, 49, 199–222. [CrossRef] [PubMed]
- 9. Kamada, R.; Kudoh, F.; Ito, S.; Tani, I.; Janairo, J.I.B.; Omichinski, J.G.; Sakaguchi, K. Metal-dependent ser/thr protein phosphatase ppm family: Evolution, structures, diseases and inhibitors. *Pharmacol. Ther.* **2020**, *215*, 107622. [CrossRef]
- 10. Merlot, S.; Gosti, F.; Guerrier, D.; Vavasseur, A.; Giraudat, J. The abi1 and abi2 protein phosphatases 2c act in a negative feedback regulatory loop of the abscisic acid signalling pathway. *Plant J.* **2001**, *25*, 295–303. [CrossRef]
- Ma, Y.; Szostkiewicz, I.; Korte, A.; Moes, D.; Yang, Y.; Christmann, A.; Grill, E. Regulators of pp2c phosphatase activity function as abscisic acid sensors. *Science* 2009, 324, 1064–1068. [CrossRef]
- 12. Sakata, Y.; Komatsu, K.; Taji, T.; Tanaka, S. Role of pp2c-mediated aba signaling in the mossphyscomitrella patens. *Plant Signal. Behav.* **2014**, *4*, 887–889. [CrossRef]
- 13. Soon, F.; Ng, L.; Zhou, X.E.; West, G.M.; Kovach, A.; Tan, M.H.E.; Suino-Powell, K.M.; He, Y.; Xu, Y.; Chalmers, M.J.; et al. Molecular mimicry regulates aba signaling by snrk2 kinases and pp2c phosphatases. *Science* **2012**, *335*, 85–88. [CrossRef]
- Moreno-Alvero, M.; Yunta, C.; Gonzalez-Guzman, M.; Lozano-Juste, J.; Benavente, J.L.; Arbona, V.; Menéndez, M.; Martinez-Ripoll, M.; Infantes, L.; Gomez-Cadenas, A.; et al. Structure of ligand-bound intermediates of crop aba receptors highlights pp2c as necessary aba co-receptor. *Mol. Plant* 2017, 10, 1250–1253. [CrossRef]
- Umezawa, T.; Sugiyama, N.; Mizoguchi, M.; Hayashi, S.; Myouga, F.; Yamaguchi-Shinozaki, K.; Ishihama, Y.; Hirayama, T.; Shinozaki, K. Type 2c protein phosphatases directly regulate abscisic acid-activated protein kinases in *Arabidopsis. Proc. Natl. Acad. Sci. USA* 2009, 106, 17588–17593. [CrossRef]
- Vlad, F.; Rubio, S.; Rodrigues, A.; Sirichandra, C.; Belin, C.; Robert, N.; Leung, J.; Rodriguez, P.L.; Laurière, C.; Merlot, S. Protein phosphatases 2c regulate the activation of the snf1-related kinase ost1 by abscisic acid in *Arabidopsis*. *Plant Cell* 2009, 21, 3170–3184. [CrossRef]
- 17. Fujii, H.; Chinnusamy, V.; Rodrigues, A.; Rubio, S.; Antoni, R.; Park, S.; Cutler, S.R.; Sheen, J.; Rodriguez, P.L.; Zhu, J. In vitro reconstitution of an abscisic acid signalling pathway. *Nature* **2009**, *462*, 660–664. [CrossRef] [PubMed]
- Yoshida, T.; Fujita, Y.; Sayama, H.; Kidokoro, S.; Maruyama, K.; Mizoi, J.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Areb1, areb2, and abf3 are master transcription factors that cooperatively regulate abre-dependent aba signaling involved in drought stress tolerance and require aba for full activation. *Plant J.* 2010, *61*, 672–685. [CrossRef] [PubMed]
- 19. Kim, S.Y. The role of abf family bzip class transcription factors in stress response. Physiol. Plant. 2005, 126, 519–527. [CrossRef]
- 20. Sheen, J. Mutational analysis of protein phosphatase 2c involved in abscisic acid signal transduction in higher plants. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 975–980. [CrossRef]

- 21. Rodriguez, P.L. Protein phosphatase 2c (pp2c) function in higher plants. Plant Mol. Biol. 1998, 38, 919–927. [CrossRef] [PubMed]
- Sugimoto, H.; Kondo, S.; Tanaka, T.; Imamura, C.; Muramoto, N.; Hattori, E.; Ogawa, K.; Mitsukawa, N.; Ohto, C. Overexpression of a novel *Arabidopsis* pp2c isoform, atpp2cf1, enhances plant biomass production by increasing inflorescence stem growth. *J. Exp. Bot.* 2014, 65, 5385–5400. [CrossRef]
- Nishimura, N.; Yoshida, T.; Kitahata, N.; Asami, T.; Shinozaki, K.; Hirayama, T. Aba-hypersensitive germination1 encodes a protein phosphatase 2c, an essential component of abscisic acid signaling in *Arabidopsis* seed. *Plant J.* 2007, *50*, 935–949. [CrossRef] [PubMed]
- 24. He, Z.; Wu, J.; Sun, X.; Dai, M. The maize clade a pp2c phosphatases play critical roles in multiple abiotic stress responses. *Int. J. Mol. Sci.* **2019**, *20*, 3573. [CrossRef]
- Yu, X.; Han, J.; Li, L.; Zhang, Q.; Yang, G.; He, G. Wheat pp2c-a10 regulates seed germination and drought tolerance in transgenic Arabidopsis. Plant Cell Rep. 2020, 39, 635–651. [CrossRef] [PubMed]
- Wang, G.; Sun, X.; Guo, Z.; Joldersma, D.; Guo, L.; Qiao, X.; Qi, K.; Gu, C.; Zhang, S. Genome-wide identification and evolution of the pp2c gene family in eight Rosaceae species and expression analysis under stress in *Pyrus bretschneideri*. *Front. Genet.* 2021, 12, 770014. [CrossRef]
- 27. Zhu, J.K. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 2002, 53, 247–273. [CrossRef]
- Belin, C.; Reichheld, J.P. Redox and acetylation coordinate aba-independent osmotic stress response. *Mol. Plant* 2022, 15, 583–585. [CrossRef]
- Yoo, S.D.; Cho, Y.H.; Sheen, J. Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2007, 2, 1565–1572. [CrossRef]
- 30. Aguilar, M.L. The role of abscisic acid in controlling leaf water loss, survival and growth of micropropagated tagetes erecta plants when transferred directly to the field. *J. Exp. Bot.* **2000**, *51*, 1861–1866. [CrossRef]
- Hirayama, T.; Shinozaki, K. Perception and transduction of abscisic acid signals: Keys to the function of the versatile plant hormone aba. *Trends Plant Sci.* 2007, *12*, 343–351. [CrossRef] [PubMed]
- Lind, C.; Dreyer, I.; López-Sanjurjo, E.J.; von Meyer, K.; Ishizaki, K.; Kohchi, T.; Lang, D.; Zhao, Y.; Kreuzer, I.; Al-Rasheid, K.A.S.; et al. Stomatal guard cells co-opted an ancient aba-dependent desiccation survival system to regulate stomatal closure. *Curr. Biol.* 2015, 25, 928–935. [CrossRef] [PubMed]
- Tischer, S.V.; Wunschel, C.; Papacek, M.; Kleigrewe, K.; Hofmann, T.; Christmann, A.; Grill, E. Combinatorial interaction network of abscisic acid receptors and coreceptors from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 2017, *114*, 10280–10285. [CrossRef] [PubMed]
- Tovar-Mendez, A.; Miernyk, J.A.; Hoyos, E.; Randall, D.D. A functional genomic analysis of *Arabidopsis thaliana* pp2c clade D. *Protoplasma* 2014, 251, 265–271. [CrossRef]
- Chen, C.; Yu, Y.; Ding, X.; Liu, B.; Duanmu, H.; Zhu, D.; Sun, X.; Cao, L.; Zaib-Un-Nisa; Li, Q.; et al. Genome-wide analysis and expression profiling of pp2c clade d under saline and alkali stresses in wild soybean and *Arabidopsis*. *Protoplasma* 2018, 255, 643–654. [CrossRef]
- 36. Khan, N.; Ke, H.; Hu, C.M.; Naseri, E.; Haider, M.S.; Ayaz, A.; Amjad, K.W.; Wang, J.; Hou, X. Genome-wide identification, evolution, and transcriptional profiling of pp2c gene family in *Brassica rapa*. *BioMed Res. Int.* **2019**, 2019, 2965035. [CrossRef]
- 37. Yu, X.; Han, J.; Wang, E.; Xiao, J.; Hu, R.; Yang, G.; He, G. Genome-wide identification and homoeologous expression analysis of pp2c genes in wheat (*Triticum aestivum L.*). *Front. Genet.* **2019**, *10*, 561. [CrossRef] [PubMed]
- Shazadee, H.; Khan, N.; Wang, J.; Wang, C.; Zeng, J.; Huang, Z.; Wang, X. Identification and expression profiling of protein phosphatases (pp2c) gene family in *Gossypium hirsutum L. Int. J. Mol. Sci.* 2019, 20, 1395. [CrossRef] [PubMed]
- Cutler, S.R.; Rodriguez, P.L.; Finkelstein, R.R.; Abrams, S.R. Abscisic acid: Emergence of a core signaling network. *Annu. Rev. Plant Biol.* 2010, 61, 651–679. [CrossRef] [PubMed]
- 40. Ren, H.; Park, M.Y.; Spartz, A.K.; Wong, J.H.; Gray, W.M. A subset of plasma membrane-localized pp2c.d phosphatases negatively regulate saur-mediated cell expansion in *Arabidopsis*. *PLoS Genet.* **2018**, *14*, e1007455. [CrossRef]
- 41. Moes, D.; Himmelbach, A.; Korte, A.; Haberer, G.; Grill, E. Nuclear localization of the mutant protein phosphatase abi1 is required for insensitivity towards aba responses in *Arabidopsis*. *Plant J.* **2008**, *54*, 806–819. [CrossRef] [PubMed]
- Papacek, M.; Christmann, A.; Grill, E. Interaction network of aba receptors in grey poplar. *Plant J.* 2017, 92, 199–210. [CrossRef] [PubMed]
- Lee, S.C.; Lim, C.W.; Lan, W.; He, K.; Luan, S. Aba signaling in guard cells entails a dynamic protein–protein interaction relay from the pyl-rcar family receptors to ion channels. *Mol. Plant* 2013, *6*, 528–538. [CrossRef] [PubMed]
- 44. Wang, Y.; Yu, H.; Zhang, Y.; Lai, C.; She, Y.; Li, W.; Fu, F. Interaction between abscisic acid receptor pyl3 and protein phosphatase type 2c in response to aba signaling in maize. *Gene* **2014**, *549*, 179–185. [CrossRef] [PubMed]
- Wang, Y.; Fu, F.; Yu, H.; Hu, T.; Zhang, Y.; Tao, Y.; Zhu, J.; Zhao, Y.; Li, W. Interaction network of core aba signaling components in maize. *Plant Mol. Biol.* 2018, 96, 245–263. [CrossRef]
- Zhao, Y.; Chan, Z.; Gao, J.; Xing, L.; Cao, M.; Yu, C.; Hu, Y.; You, J.; Shi, H.; Zhu, Y.; et al. Aba receptor pyl9 promotes drought resistance and leaf senescence. *Proc. Natl. Acad. Sci. USA* 2016, 113, 1949–1954. [CrossRef]
- Yang, J.; Wang, M.; Zhou, S.; Xu, B.; Chen, P.; Ma, F.; Mao, K. The aba receptor gene mdpyl9 confers tolerance to drought stress in transgenic apple (*Malus domestica*). *Environ. Exp. Bot.* 2022, 194, 104695. [CrossRef]

- 48. He, Z.; Zhong, J.; Sun, X.; Wang, B.; Terzaghi, W.; Dai, M. The maize aba receptors zmpyl8, 9, and 12 facilitate plant drought resistance. *Front. Plant Sci.* **2018**, *9*, 422. [CrossRef]
- 49. Fujita, Y.; Yoshida, T.; Yamaguchi-Shinozaki, K. Pivotal role of the areb/abf-snrk2 pathway in abre-mediated transcription in response to osmotic stress in plants. *Physiol. Plant.* **2013**, *147*, 15–27. [CrossRef]
- Li, Y.; Sun, H.; Wang, Z.; Duan, M.; Huang, S.; Yang, J.; Huang, J.; Zhang, H. A novel nuclear protein phosphatase 2c negatively regulated by abl1 is involved in abiotic stress and panicle development in rice. *Mol. Biotechnol.* 2013, 54, 703–710. [CrossRef]
- 51. Singh, A.; Jha, S.K.; Bagri, J.; Pandey, G.K. Aba inducible rice protein phosphatase 2c confers aba insensitivity and abiotic stress tolerance in *Arabidopsis*. *PLoS ONE* **2015**, *10*, e125168. [CrossRef] [PubMed]
- 52. Hetherington, A.M. Guard cell signaling. Cell 2001, 107, 711–714. [CrossRef]
- 53. Fujita, Y.; Fujita, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. Aba-mediated transcriptional regulation in response to osmotic stress in plants. *J. Plant Res.* 2011, 124, 509–525. [CrossRef]
- Lian, C.; Li, Q.; Yao, K.; Zhang, Y.; Meng, S.; Yin, W.; Xia, X. Populus trichocarpa ptnf-ya9, a multifunctional transcription factor, regulates seed germination, abiotic stress, plant growth and development in *Arabidopsis. Front. Plant Sci.* 2018, 9, 954. [CrossRef] [PubMed]
- 55. Rovira, A.; Sentandreu, M.; Nagatani, A.; Leivar, P.; Monte, E. The sequential action of mida9/pp2c.d1, pp2c.d2, and pp2c.d5 is necessary to form and maintain the hook after germination in the dark. *Front. Plant Sci.* **2021**, *12*, 636098. [CrossRef] [PubMed]
- 56. Chang, S.; Puryear, J.; Cairney, J. A simple and efficient method for isolating rna from pine trees. *Plant Mol. Biol. Rep.* **1993**, *11*, 113–116. [CrossRef]
- Kumar, S.; Nei, M.; Dudley, J.; Tamura, K. Mega: A biologist-centric software for evolutionary analysis of dna and protein sequences. *Brief. Bioinform.* 2008, *9*, 299–306. [CrossRef]
- Clough, S.J.; Bent, A.F. Floral dip: A simplified method for agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 1998, 16, 735–743. [CrossRef]
- Velikova, V.; Yordanov, I.; Edreva, A. Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Sci.* 2000, 151, 59–66. [CrossRef]
- Paiano, A.; Margiotta, A.; De Luca, M.; Bucci, C. Yeast two-hybrid assay to identify interacting proteins. *Curr. Protoc. Protein Sci.* 2019, 95, e70. [CrossRef]
- 61. Wang, H.H.; Qiu, Y.; Yu, Q.; Zhang, Q.; Li, X.; Wang, J.; Li, X.; Zhang, Y.; Yang, Y. Close arrangement of cark3 and pmeil affects aba-mediated pollen sterility in *Arabidopsis thaliana*. *Plant Cell Environ*. **2020**, *43*, 2699–2711. [CrossRef] [PubMed]
- Waadt, R.; Schmidt, L.K.; Lohse, M.; Hashimoto, K.; Bock, R.; Kudla, J. Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative cbl/cipk complexesin planta. *Plant J.* 2008, *56*, 505–516. [CrossRef] [PubMed]