An R2R3-MYB Transcription Factor PgFLP Directly Activates *PgPIN10* to Regulate the GSA of Adventitious Root in Pomegranate

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Research article

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Abstract

Background: The self-rooted seedling is widely used in pomegranate planting industry currently; However, the root system of self-rooted seedling is shallow and poor cold resistance. Therefore, the study of the molecular mechanisms of pomegranate adventitious root gravitropism is very important for developing deep-rooted pomegranate cultivars.

Results: We report the pomegranate FOUR LIPS (PgFLP) that play an key role in regulating the gravitropic set-point angle of pomegranate adventitious root in response to gravity signal. In our study, PgFLP directly regulates the transcriptional expression of *PgPIN10* by binding to its promoter, thus regulating the GSA of adventitious root in pomegranate. Additionally, the *35S::PgFLP* show stronger gravitational response than wild-type, leading to a smaller GSA in *Arabidopsis* lateral roots, indicating that PgFLP participates in regulating the GSA of adventitious root via *PgPIN10* in pomegranate.

Conclusion: Our results confirm that the transcriptional regulation of *PgPIN10* by R2R3-MYB transcription factor PgFLP in setting the gravitropic set-point angle of pomegranate adventitious root in response to gravity signal.

Background

Pomegranate (*Punica granatum* L.) is believed to originated in Afghanistan, present-day Iran, India, and Turkmenistan and other central Asia countries [36, 23]. In China, many recognized pomegranate varieties with high genetic diversity have been cultivated [33]. The self-rooted seedling is widely used in pomegranate planting industry currently. However, the development of gravitropic set-point angle (GSA) between self-rooted seedling and pomegranate seedling is a great difference, leading to the adventitious root of pomegranate was poor in shallowness and solidity, resulted in poor cold resistance [28].

The plant hormone auxin is explicit for a common integrator to many environmental and endogenous signals regulating lateral root formation [12]. In the lateral root cap, the auxin stimulating prebranch site formation [32]. Subsequently, the dynamic auxin flows orchestrated lateral root patterning and morphogenesis [3]. In addition, it interacts with the surrounding tissue in a complex manner [10, 16, 19, 26]. The gravitropic set-point angle can be defined by orientation of plant growth with respect to the gravity vector [5]. In addition, auxin controls gravitropic set-point angle of lateral branches through auxin signaling pathway dependent on TIR1/AFB-Aux/IAA-ARF in the higher plant [22].

MYB TFs have been found to regulate plant development and metabolism [7]. In *Arabidopsis*, the two R2R3-MYB proteins, MYB88 and FOUR LIPS (FLP), can bind directly to the promoters of *PINs* genes that harbour an [A/T/G][A/T/G]C[C/G][C/G] motif [31]. FLP and MYB88 have been found to play redundantly role in limiting terminal divisions in *Arabidopsis*. Compared with *flp* single mutants, the stomatal defects of *flp-1myb88* double mutants are more severe [11]. In addition, FLP and MYB88 have been found to can regulate root gravitropism [27], the late stages of stomatal development [11], cold hardiness [30], female reproductive development [17], and guard mother cell proliferation [11, 31]. The *PINs* have been found to

conductive to nearly every step of auxin fluxes [3, 18, 35, 20]. Following the primary roots are stimulated by gravity, the PINs have been repolarized [21, 29], the auxin flux redirection to the lower side of roots, leading to root tip bending [8, 9, 22, 25]. In addition, the polar auxin transport is beneficial to the root bending under obstacle avoidance [13]. The auxin distribution patterns have been predicted and explained through auxin transport [2, 6, 1]. The *AtPIN3* shows remarkably dynamic expression during the root gravitropism and patterning of stomatal complexes in *Arabidopsis* [14, 27]. However, how regulators regulate *PIN* expression combining with auxin signalling pathway during root development is nothing known in pomegranate. We investigated the mechanisms regulating *PgPIN10* expression during pomegranate adventitious root gravitropism.

The regulation of root gravitropism in pomegranate is very little known currently. In our study, PgFLP participates in regulating the gravitropic set-point angle of pomegranate adventitious root. PgFLP directly regulates the transcriptional expression of *PgPIN10* by binding to its promoter, thus regulating the GSA of adventitious root in pomegranate. Our results confirm that the transcriptional regulation of *PgPIN10* by PgFLP in setting the gravitropic set-point angle of pomegranate adventitious root in response to gravity signal.

Results

The expression pattern of *PgPINs* in different rootedness pomegranate self-rooted seedling adventitious root

Two different rootedness pomegranate self-rooted seedling are screened in this study, compared with each other, the two self-rooted seedling show different rootedness adventitious roots branch angle (Table 1). The two self-rooted seedling provide appropriate material development for the study of GSA. We had screened the shallow-rooted pomegranate breed 'Taishanhong'(TSH) and the deep-rooted pomegranate breed: 'Lanbaoshi'(LBS) (Fig. 1a). The *PINs* have been found to conducive to nearly every step of auxin fluxes [3, 18, 35, 20] and the auxin distribution patterns have been predicted and explained through auxin transport [2, 6, 1]. In this study, the expression of *PgPIN1* and *PgPIN10* showed higher in LBS than TSH (Fig. 1b). We also investigated the expression of other *PgPINs*, *PgPIN3* and *PgPIN7* showed lower in LBS than TSH (Additional file 1: Figure S1). These results indicated that *PgPINs* may participate in the regulation of GSA in pomegranate adventitious root.

Molecular cloning of *PgFLP*

To further characterize the function of *PgPINs* in pomegranate, we performed the Y1H screening via pomegranate cDNA library. Eventually, we used the promoter sequences of *PgPIN10* as baits to obtained a cDNA fragment of PgFLP. To identify the pomegranate *PgFLP* that is involved in adventitious root gravitropism, a genome-wide analysis was performed. Comparison of amino acid sequences of PgFLP with their close homologs, the PgFLP protein is highly conserved identity to R2R3-MYB protein (Fig. 2a). To understand the evolutionary relationship of PgFLP, using the neighbor-joining (NJ) method [24], we constructed a phylogenetic tree. As a result, We found that PgFLP had been clustered within the R2R3-

MYB clade including AtFLP, MdFLP and others for which root gravitropism have been identified. Within the R2R3-MYB clade, PgFLP is closely associated with AtFLP, which regulate lateral root gravitropism, indicating that PgFLP may participate in the regulation of GSA in pomegranate adventitious root (Fig. 2b).

Subcellular localization analysis of PgFLP

To further characterize the function of PgFLP in pomegranate, we used the coding sequence of *PgFLP* and GFP reporter to constructed a *35S::PgFLP-GFP* translational fusion. We found that in contrast to *35S::GFP* where a fluorescent signal was observed throughout the whole epidermal cell, the *35S::PgFLP-GFP* showed a signal in nucleus (Fig. 3a-b), suggesting that as a transcription factor, PgFLP may be involved in pomegranate adventitious root gravitropism.

***PgFLP* expression pattern in pomegranate**

To further determine the function of PgFLP, we performed the qRT-PCR to determine the expression patterns of *PgFLP* in pomegranate. We found that the expressing *PgFLP* were detected in various organs of pomegranate, especially higher expressing in the leaves and the fruit peels (Fig. 4a). The auxin participate in triggering lateral root development [4], we investigated whether *PgFLP* expression is induced by auxin. The expression of *PgFLP* was rapidly up-regulated in auxin-treated roots (Fig. 4b). We also investigated the expression of *PgMYB88* was not significantly induced by auxin (Additional file 1: Figure S2b). Subsequently, we found that the transcripts of *PgFLP* in LBS was increased (~2-fold) upon the TSH (Fig. 4c). We also investigated the IAA content in different rootedness pomegranate self-rooted seedling and found that the level of IAA was slightly increased in LBS compared to that in TSH on shoot cutting at 30 d and 40 d (Fig. 4d). These results indicated that *PgFLP* plays an key role in pomegranate adventitious root gravitropism.

PgFLP directly regulate *PgPIN10* transcription

To further characterize the mechanism of PgFLP to regulate GSA of adventitious root in pomegranate, we performed the Y1H assays. Y1H assays showed that PgFLP could interact with the promoter of *PgPIN10* to activate its expression (Fig. 5a). To further investigate whether PgFLP could activate the transcriptional expression of *PgPIN10* in planta, we conducted a transient expression assay. As a result, the *proPgPIN10::GUS* plus PgFLP showed significantly higher GUS activity in tobacco leaves than the control (Fig. 5b-d). These results showed that PgFLP could activate the expression of *PgPIN10* in pomegranate.

It is well established that FLP can bind directly to the promoters of *PINs* genes that harbour an [A/T/G][A/T/G]C[C/G][C/G] motif [31]. We found that there was two potential binding sites including AGCGG and TACCC in the promoter region of *PgPIN10* (Fig. 5e and Additional file 1: Figure S3). To further determine if PgFLP could directly bind to these binding sites in plants, we performed Y1H assays. We found that PgFLP could interact with FBS (AGCGG). On the other hand, PgFLP could not interact with the mutated

FBS motif (mFBS) (Fig. 5f). These data demonstrate that PgFLP directly regulate *PgPIN10* transcription by binding to the AGCGG motif.

Ectopic overexpression of *PgFLP* in *Arabidopsis* seedlings results in a smaller GSA of lateral root

As it is difficult to obtain transgenic pomegranate plants overexpressing *PgFLP*, *PgFLP* was ectopically expressed in *Arabidopsis* to further characterize if PgFLP functions in the regulation GSA of pomegranate self-rooted seedling adventitious root in planta. Three independent transgenic lines, OE3, OE4 and OE7, were chosen for further analysis (Fig. 6a). To investigate phenotypes of the WT and *PgFLP* overexpression lines grown for 21 days on MS media, we found that *PgFLP* (OX-3, OX-4, OX-7) (Fig. 6b-e), exhibited a smaller GSA and grew downwards faster than WT. For example, over 35% of *PgFLP*-OX lateral root increased within a 30-50° range, meanwhile, within a 50-70° range, over 30% of lateral root was decreased (Fig. 6f). These results indicated that PgFLP functions in the regulation GSA of pomegranate self-rooted seedling adventitious root in planta.

Discussion

PgFLP is involved in the pomegranate self-rooted seedling adventitious root gravitropism

The self-rooted seedling is widely used in pomegranate planting industry currently. However, the development of gravitropic set-point angle (GSA) between self-rooted seedling and pomegranate seedling is a great difference, leading to the adventitious root of pomegranate was poor in shallowness and solidity, resulted in poor cold resistance [28]. FLP is angle-inducible gene in response to gravity in *Arabidopsis* and apple [27, 28]. We found that PgFLP had been clustered within the R2R3-MYB clade including AtFLP, MdFLP and others for which root gravitropism have been identified. Within the R2R3-MYB clade, PgFLP is closely associated with AtFLP, which regulate lateral root gravitropism, indicating that PgFLP may participate in the regulation of GSA in pomegranate adventitious root (Fig. 2b).

The *PIN*s have been found to conducive to nearly every step of auxin fluxes [3, 18, 35, 20] and the auxin distribution patterns have been predicted and explained through auxin transport [2, 6, 1]. FLP and MYB88 have been found to can regulate root gravitropism [27], the late stages of stomatal development [11], cold hardiness [30], female reproductive development [17], and guard mother cell proliferation [11, 31]. To further understand the function of *PgFLP*, the expression of *PgFLP* was rapidly up-regulated in auxin-treated roots (Fig. 4b) and the transcripts of *PgFLP* in LBS was increased (~2-fold) upon the TSH (Fig. 4c). We also investigated the IAA content in different rootedness pomegranate self-rooted seedling and found that the level of IAA was slightly increased in LBS compared to that in TSH on shoot cutting at 30 d and 40 d (Fig. 4d). *PgFLP* (OX-3, OX-4, OX-7) (Fig. 6b-e), exhibited a smaller GSA and grew downwards faster than WT. In addition, *PgMYB88* transcripts were detected in various pomegranate organs, but the expression of *PgMYB88* was not significantly induced by auxin (Additional file 1: Figure S2a-b). We also found that the transcripts of *PgMYB88* in LBS was increased (~3-fold) upon the TSH (Additional file 1: Figure S2c). Whether *PgMYB88* is also involved in transcriptional regulation of adventitious root gravitropism in pomegranate requires further investigation. These results suggested that PgFLP

participates in regulating the GSA of adventitious root via the basal expression of *PgPIN10* in pomegranate.

The *PgFLP* is participated in the regulatory network during the pomegranate adventitious root gravitropism

It is well established that FLP can bind directly to the [A/T/G][A/T/G]C[C/G][C/G] motif [31]. Because PgFLP is closely associated with AtFLP, which regulate lateral root gravitropism, and PgFLP functions in the regulation GSA of pomegranate self-rooted seedling adventitious root in planta (Fig. 6). Y1H experiments showed that PgFLP can interact with FBS (AGCGG). We also found the expression of *PgFLP* was rapidly up-regulated in auxin-treated roots (Fig. 4b), indicating that the function of PgFLP may be more diverse compared with *FLP* in *Arabidopsis*.

We found that FLP can activate the expression of *PIN3* and *PIN7* in *Arabidopsis* [27]. In our study, we found that PgFLP could activate the expression of *PgPIN10* in pomegranate (Fig. 5) and PgFLP could interact with FBS (AGCGG) (Fig. 5f). These data demonstrate that PgFLP directly regulate *PgPIN10* transcription by binding to the AGCGG motif. How PgFLP is participated in the feed-forward transcriptional regulation of *PgPIN10* during the pomegranate adventitious root gravitropism needs further investigation.

In our study, a model of PgFLP in response to gravity signal during pomegranate self-rooted seedling adventitious root gravitropism was proposed (Fig. 7). Under gravity, PgFLP directly regulates the expression of *PgPIN10* by binding to the AGCGG motif to regulate GSA. On the other hand, other *PgPINs* may also in response to IAA affecting auxin reflux to regulate GSA. Overall, our results confirm that the transcriptional regulation of *PgPIN10* by R2R3-MYB transcription factor PgFLP in setting the gravitropic set-point angle of pomegranate adventitious root in response to gravity signal.

Conclusions

An R2R3-MYB transcription factor PgFLP directly activates *PgPIN10* by binding to the AGCGG motif of its promoter region to regulate the GSA of adventitious root in pomegranate. Overall, our results confirm that the transcriptional regulation of *PgPIN10* by PgFLP in setting the gravitropic set-point angle of pomegranate adventitious root in response to gravity signal.

Methods

Plant materials and growth conditions

The deep-rooted pomegranate breed: 'Lanbaoshi'(LBS) and shallow-rooted pomegranate breed: 'Taishanhong'(TSH), provided by Professor Chuanduo Shi (Shandong Institute of Pomology), were for the study of GSA. The roots of 'Taishanhong'(TSH) were used for gene cloning. *Arabidopsis thaliana* (ecotype 'Columbia'), provided by Professor Xiang Shen (Shandong Agricultural University), was for

genetic transformation. The *Arabidopsis* seeds were grown on MS medium at 22°C for 16 hours of light / 8 hours of dark.

RNA extraction and qRT-PCR analysis

We used Total RNA Isolation System to extract total RNA from pomegranate tissues. Then, an appropriate amount of total RNA was taken to synthesize cDNA with the PrimeScript®1st Strand cDNA Synthesis Kit (Takara, Japan). The qRT-PCR was performed as previously described [15, 28]. The relative expression level of each target gene was normalized to that of the *ACTIN* gene. Three biological replicates were performed for each analysis. Details primers used in this study are shown in Additional file 2: Table S1.

Sequence and phylogenetic tree analysis

The corresponding amino acid sequences were obtained from the NCBI nucleotide database and aligned using ClustalX 1.81 and DNAMAN software. The corresponding protein accession numbers: *PgFLP*, *Punica granatum* XP_031400371.1; *PgMYB88*, *Punica granatum* XP_031386277.1; *MdMYB88*, *Malus domestica* ASW25823.1; *MdFLP*, *Malus domestica* XP_017192102.1; *MdMYB124*, *Malus domestica* ASW25824.1; *AtMYB88*, *Arabidopsis thaliana* NP_001030957.1; *AtMYB124 (AtFLP)*, *Arabidopsis thaliana* NP_563948.1; *FvMYB a*, *Fragaria vesca* XP_004297324.1; *FvMYB b*, *Fragaria vesca* XP_011463081.1; *VvMYB a*, *Vitis vinifera* XP_010652303.1; *VvMYB b*, *Vitis vinifera* XP_010652298.1; *OsMYB a*, *Oryza sativa* EEC82494.1; *OsMYB b*, *Oryza sativa* NP_001060344.1. The phylogenetic tree was constructed according to Tamura et al. [24].

Yeast one-hybrid assays

Y187 (Clontech) was used for Y1H assays. The *PgFLP* was recombined into the pGADT7 vector to obtain the AD-*PgFLP* plasmid, and the *PgPIN10* promoter was cloned into the pHIS2 vector. Yeast Y187 cells containing different combinations of recombinant vectors were placed on medium SD/-Leu-Trp-His with different concentration of 3-AT for detection.

GUS transient assays

The tobacco leaves were used to conduct transient expression assays. The promoter of *PgPIN10* was cloned into pCAMBIA1300-GUS to obtain the *proPgPIN10::GUS* recombinant vector. The coding sequence of *PgFLP* were inserted into pRI101 plant transformation vector downstream of 35S promoter. The corresponding combinations were co-injected into tobacco leaves according to previously described [28]. The tobacco of the experimental group was cultured normally for 2 days. Then, we detected *GUS* activity as previously described [28].

The construction of *PgFLP* overexpression vector and genetic transformation

The complete *PgFLP* coding region was integrated into the pRI101 plant transformation vector downstream of 35S promoter. The *35S::PgFLP* vector was transformed into *Arabidopsis* plants [27]. The

seeds of the transgenic *Arabidopsis* plants were individually harvested. Homozygous transgenic lines were used for further investigation.

Quantification of plant hormones

The pomegranate self-rooted seedling adventitious roots were immediately frozen with liquid nitrogen. Then, we used ELISA (Enzyme-Linked Immuno Sorbent Assay) to measure hormones IAA according to Zhang et al [34].

Measurement of gravity set-point angle

Transgenic *Arabidopsis thaliana* roots were used to measure GSA. The GSA angles were divided into 0-30°, 30-50°, 50-70°, 70-90° and 90-110°. The Image J software was used to measure GSA. Experiments were repeated independently three times. Statistical significance: **P < 0.01.

Abbreviations

FLP, FOUR LIPS; PIN, PIN-FORMED; IAA, indole-3-acetic acid; GSA, gravitropic set-point angle; TFs, transcription factors; TSH, 'Taishanhong'; LBS 'Lanbaoshi'; GFP, green fluorescent protein; MS, Murashige and Skoog; WT, wild-type; GUS, β -glucuronidase; qRT-PCR, quantitative reverse transcription-PCR; Y1H, yeast one-hybrid.

Declarations

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Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YLY conceived the project. YLY, JHT and ZHW designed the experiments. ZHW performed most of the experiments, and ZHW wrote the paper. JLL helped with the transient expression assays; JLL, XMY, HXT, LJF and FW helped with the phenotypic observations and the paper revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 The angle between adventitious root and gravity (°)

Root architecture	No. of Adventitious roots	Cuttings	X<30		30≤X≤60		X≥60	
			No. of roots	Percent	No. of roots	Percent	No. of roots	Percent
Deep roots	47	LBS	11	23.40%	30	63.83%	6	12.77%
Shallow roots	52	TSH	4	7.69%	28	53.85%	20	38.46%
X, represent as the angle between adventitious root and gravity; LBS, ‘Lanbaoshi’; TSH, ‘Taishanhong’.								

Figures

A **B**

Figure 1

The expression pattern of PgPINs in different rootedness pomegranate self-rooted seedling adventitious root. (a) The shallow-rooted pomegranate breed: 'Taishanhong'(TSH). The deep-rooted pomegranate breed: 'Lanbaoshi'(LBS). (b) Transcript levels of PgPIN1 and PgPIN10 in pomegranate self-rooted seedling adventitious root. Scale bars = 1cm. Error bars are means ($\pm$ SD) of three technical replicates. All experiments were repeated three to four times with similar results, and one representative biological repeat was shown.

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Figure 2

Comparative analysis of PgFLP and its homologs in other plant species. (a) Comparison of amino acid sequences of PgFLP with its close homologs. The accession numbers for the aligned proteins are: PgFLP, *Punica granatum* XP_031400371.1; PgMYB88, *Punica granatum* XP_031386277.1; MdMYB88, *Malus domestica* ASW25823.1; MdFLP, *Malus domestica* XP_017192102.1; MdMYB124, *Malus domestica* ASW25824.1; AtMYB88, *Arabidopsis thaliana* NP_001030957.1; AtMYB124 (AtFLP), *Arabidopsis thaliana*

NP_563948.1; FvMYB a, *Fragaria vesca* XP_004297324.1; FvMYB b, *Fragaria vesca* XP_011463081.1; VvMYB a, *Vitis vinifera* XP_010652303.1; VvMYB b, *Vitis vinifera* XP_010652298.1; OsMYB a, *Oryza sativa* EEC82494.1; OsMYB b, *Oryza sativa* NP_001060344.1. (b) Phylogenetic tree of PgFLP and their close homologs in plant species. The protein identities are the same as in A, red circle represents PgFLP.

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Figure 3

Subcellular localization of PgFLP protein in tobacco leaf cells. (a)Schematic illustration of 35S::GFP (empty vector) and 35S::PgFLP-GFP (fusion expression vector). (b) Transient expression of 35S::PgFLP-GFP and 35S::GFP in tobacco leaf cells. The cells expressing 35S::PgFLP-GFP showed fluorescence signal in the nucleus. Bars=50 μ m.

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Figure 4

The expression pattern of PgFLP and IAA content in different rootedness pomegranate self-rooted seedling adventitious root. (a) The expression of PgFLP in seeds (S), roots (R), leaves (L), flowers (F) and fruit peels (Fp) of pomegranate. (b) Relative expression analysis of PgFLP in roots of TSH treated with auxin (10 mM NAA) during 0h, 0.5h, 2h, 4h, 6h, 12h time course. (c) Transcript levels of PgFLP in pomegranate self-rooted seedling adventitious root. (d) The hormone IAA content in pomegranate self-rooted seedling adventitious root. Error bars are means ($\pm$ SD) of three technical replicates. All experiments were repeated three to four times with similar results, and one representative biological repeat was shown.

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Figure 5

PgFLP directly bound to the PgPIN10 promoter. (a) Yeast-one-hybrid assays showed PgFLP could bind to the promoter of PgPIN10. The positive colonies were screened on SD/-Trp-Leu-His containing an optimal concentration of 3-AT (60, 80, 100 mM). (b) The effector construct (35S::PgFLP) and the reporter construct (*proPgPIN10::GUS*) were used for activity assays. (c) PgFLP promoted the expression of PgPIN10 in tobacco leaves by GUS activity assays. (d) The relative expression of GUS gene in (c). All gene expression analyses were performed with at least three biological replicates. Error bars represented $\pm$ SE. Statistical significance: ** $P < 0.01$. (e) The location of FBS (AGCGG) motif on the PgPIN10 gene promoter. The position 0 on the promoter of PgPIN10 represented the translational start site (ATG). (f) Y1H assay showing PgFLP can bind to the FBS of structural PgPIN10 but not to the mutation sequence of this motif.

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Figure 6

The role of PgFLP in setting lateral root GSA. (a) PgFLP expression in WT and transgenic Arabidopsis seedlings. RNA was prepared from seedlings. Error bars are means ($\pm$ SD) of three technical replicates. All experiments were repeated three to four times with similar results, and one representative biological repeat was shown. (b-e) Phenotypes of the WT and PgFLP overexpression lines grown for 21 days on MS media. OE-3 lateral roots (c), OE-4 lateral roots (d), OE-7 lateral roots (e), exhibit a smaller GSA than WT (Col) (b). Scale bars = 1 cm. (f) Distributions of lateral root GSA. Compared with WT (Col) lateral roots, 35S::PgFLP showed a smaller GSA due to its more sensitive gravitropic responses. Error bars mean $\pm$ SE. Statistical significance: ** $P < 0.01$.

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Figure 7

Model of PgFLP in response to gravity signal during pomegranate self-rooted seedling adventitious root gravitropism. Under gravity, PgFLP directly regulates the expression of PgPIN10 by binding to the AGCGG motif to regulate GSA. In addition, other PgPINs may also in response to IAA affecting auxin reflux to regulate GSA.

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