# The Tomato 14-3-3 Protein TFT4 Modulates $H^+$ Efflux, Basipetal Auxin Transport, and the PKS5-J3 Pathway in the Root Growth Response to Alkaline Stress[1][C][W]

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Alkaline stress is a common environmental stress, in particular in salinized soils. Plant roots respond to a variety of soil stresses by regulating their growth, but the nature of the regulatory pathways engaged in the alkaline stress response (ASR) is not yet understood. Previous studies show that PIN-FORMED2, an auxin (indole-3-acetic acid [IAA]) efflux transporter, PKS5, a protein kinase, and DNAJ HOMOLOG3 (J3), a chaperone, play key roles in root $H^+$ secretion by regulating plasma membrane (PM) $H^+$-ATPases directly or by targeting 14-3-3 proteins. Here, we investigated the expression of all 14-3-3 gene family members (*TOMATO 14-3-3 PROTEIN1* [*TFT1*]–*TFT12*) in tomato (*Solanum lycopersicum*) under ASR, showing the involvement of four of them, *TFT1*, *TFT4*, *TFT6*, and *TFT7*. When these genes were separately introduced into Arabidopsis (*Arabidopsis thaliana*) and overexpressed, only the growth of *TFT4* overexpressors was significantly enhanced when compared with the wild type under stress. $H^+$ efflux and the activity of PM $H^+$-ATPase were significantly enhanced in the root tips of *TFT4* overexpressors. Microarray analysis and pharmacological examination of the overexpressor and mutant plants revealed that overexpression of *TFT4* maintains primary root elongation by modulating PM $H^+$-ATPase-mediated $H^+$ efflux and basipetal IAA transport in root tips under alkaline stress. *TFT4* further plays important roles in the PKS5-J3 signaling pathway. Our study demonstrates that *TFT4* acts as a regulator in the integration of $H^+$ efflux, basipetal IAA transport, and the PKS5-J3 pathway in the ASR of roots and coordinates root apex responses to alkaline stress for the maintenance of primary root elongation.

Alkaline soils occur commonly in terrestrial ecology, in particular in areas affected by salinity, thus contributing to one of the most widespread environmental challenges that limit agricultural productivity globally (Kawanabe and Zhu, 1991; Ge et al., 2010; Xu et al., 2012a). Worldwide, it is estimated that up to $831 \times 10^6$ ha of land is saline, and more than half of this area is alkalinized. High-pH stress limits the survival of most plants under these conditions and can be a more significant factor in reducing plant growth than the stress resulting from salinity (Guo et al., 2010). Improved understanding of the basic mechanisms of plant responses to alkaline stress is urgently needed and will aid biotechnological efforts focused on breeding suitable crops for fodder and human food on these unproductive lands.

Primary root elongation regulated by a sensory zone in the root tip plays a pivotal role in the plastic acclimation response to fluctuating soil environments (Baluška et al., 2010). The root functions simultaneously as an organ for the uptake and transport of water and nutrients and as the primary site for the perception of soil stresses. Thus, roots must be the obvious first focus in any examination of the adaptive and acclimation mechanisms underpinning the alkaline stress response. However, currently, only limited information is available on this

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particular form of stress (Degenhardt et al., 2000; Zhu, 2001; Yang et al., 2008).

Acidification of the aqueous fraction of the cell wall apoplast by $H^+$ excretion via the plasma membrane (PM) $H^+$-ATPase is a critical component of the growth-promoting effect and a key factor determining the elongation of the primary root (Moloney et al., 1981; Palmgren, 2001). Optimal primary root elongation requires the fine regulation of $H^+$-ATPase-mediated $H^+$ efflux, particularly at the root tip (Staal et al., 2011; Haruta and Sussman, 2012). Under alkaline stress, in Arabidopsis (*Arabidopsis thaliana*), PROTEIN KINASE5 (PKS5) and the chaperone DNAJ HOMOLOG3 (J3) play important roles in $H^+$ efflux by regulating the interaction between PM $H^+$-ATPase and 14-3-3 proteins (Fuglsang et al., 2007; Yang et al., 2010). Furthermore, PIN-FORMED2 (PIN2), an auxin

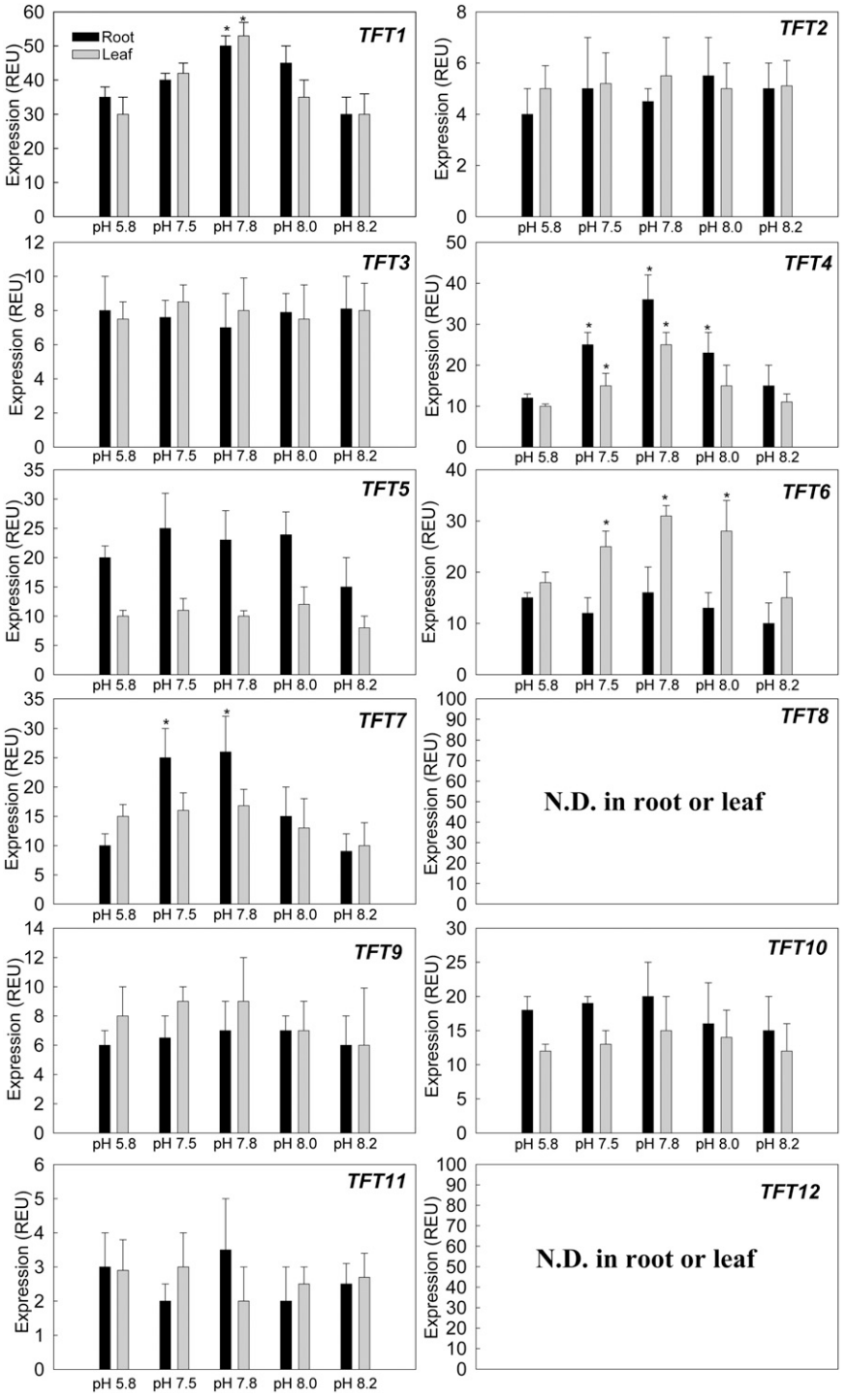


**Figure 1.** Expression of 14-3-3 gene family members in tomato plants (leaf and root) under control conditions (pH 5.8) and alkaline stress (pH 7.5, 7.8, 8.0, or 8.2) over 1 d. Relative expression levels were calculated and normalized with respect to $\alpha$-tubulin mRNA (=100 relative expression units [REU]). Asterisks represent significant differences in root or leaf under alkaline stress compared with respective control conditions ($P > 0.05$). N.D., Not detected.

(indole-3-acetic acid [IAA]) efflux transporter, is required for the acclimation of roots to alkaline stress through the modulation of $H^+$ secretion in the root tip, maintaining primary root elongation (Xu et al., 2012a). However, these mechanisms, and other physiologically relevant processes that may fine-tune root-apical responses to alkaline stress, have not been investigated in depth.

The 14-3-3 proteins are highly conserved, and nearly ubiquitous, phosphoserine-binding proteins that regulate the activities of a wide array of targets via direct protein-protein interactions (Moore and Perez, 1967; Comparot et al., 2003). In higher plants, 14-3-3 proteins are encoded by a multigene family and play important roles in regulating plant development and stress responses (Mayfield et al., 2012). Although 14-3-3 proteins in plants possess a highly conserved target-binding domain, several studies indicate that various 14-3-3 isoforms may regulate different targets or act in distinct locations under variable abiotic stresses (Sehnke et al., 2002; Xu et al., 2012b). At least 12 genes predicted to encode 14-3-3 proteins (*TOMATO 14-3-3 PROTEIN1* [*TFT1*]–*TFT12*) have been identified in tomato (*Solanum lycopersicum*; Roberts, 2003; Xu and Shi, 2006). However, little is known about the detailed actions of tomato 14-3-3 proteins in response to alkaline stress in relation to $H^+$ secretion, auxin modulation, or specific signaling pathways. Thus, in this study, we investigated the roles of tomato 14-3-3 proteins, incorporated into Arabidopsis, in root acclimation to alkaline stress and the involvement of PKS5 and J3 in modulating $H^+$ secretion and basipetal (shootward) IAA transport for maintaining primary root elongation.

## RESULTS

### Expression Profile of the Tomato 14-3-3 Gene Family in Response to Alkaline Stress

To investigate whether tomato 14-3-3 proteins are regulated by alkaline stress, we examined the expression levels of all 14-3-3 genes in both tomato leaves and roots under control conditions (pH 5.8) and under mild, realistic alkaline stresses (pH 7.5, 7.8, 8.0, and 8.2) using real-time reverse transcription (RT)-PCR (Fig. 1). Under alkaline stress, the steady-state transcript levels of most of the 14-3-3 gene family members changed little, but significant differences were observed in four genes, *TFT1*, *TFT4*, *TFT6*, and *TFT7*, in both leaves and roots. Among the alkaline stress-responsive genes, the expression levels of *TFT1* and *TFT4* were increased approximately 1.5- and 3-fold, respectively. The expression level of *TFT6* was stable in roots under alkaline stress but was about 1.5-fold higher in leaves under stress. Under alkaline stress, *TFT7* was up-regulated only in roots (about 2.5-fold).

### Performance of *TFT4*-Overexpressing Arabidopsis Plants under Alkaline Stress

Furthermore, the four alkaline stress-responsive genes (*TFT1*, *TFT4*, *TFT6*, and *TFT7*) were separately introduced into Arabidopsis and overexpressed (Supplemental Fig. S1). Only the growth of *TFT4*-overexpressing plants was significantly enhanced when compared with the wild type and other transgenic lines under alkaline stress. As shown in Supplemental Figure S2, the whole-plant dry weights of *TFT4*-overexpressing transgenic plants

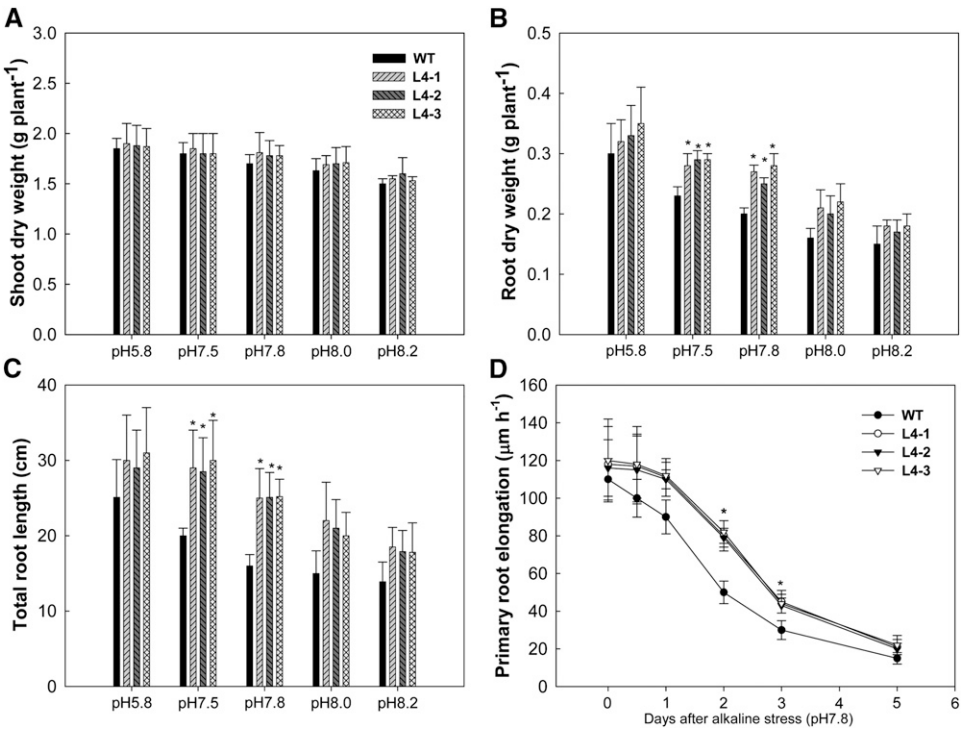


**Figure 2.** Shoot dry weight, root dry weight, total root length, and primary root elongation in wild-type (WT; Col-0) and *TFT4*-overexpressing (L4-1, L4-2, and L4-3) Arabidopsis plants under control conditions (pH 5.8) or alkaline stress (pH 7.5, 7.8, 8.0, or 8.2) over 5 d. Asterisks represent significant differences compared with the wild type ($P > 0.05$).

were significantly higher than those of the wild type (Columbia [Col-0]), but the dry weights of the overexpressing plants OE-TFT1, OE-TFT6, and OE-TFT7 were not under alkaline stress (pH 7.8 and 8.0). Moreover, no significant changes were observed in the expression levels of Arabidopsis endogenous 14-3-3 genes between the wild type and OE-TFT4 under either control conditions or alkaline stress (Supplemental Table S2). Although no significant difference in shoot dry weight was found between wild-type and *TFT4*-overexpressing plants, root dry weight and total root length in *TFT4*-overexpressing plants were significantly higher than those of the wild type exposed to alkaline stress (pH 7.5 and 7.8) for 5 d ($P < 0.05$; Fig. 2). The primary root elongation of *TFT4*-overexpressing plants was also significantly higher than that of the wild type under alkaline stress, in particular when exposed to pH 7.8 for 2 d. In localization experiments, using a p35S::TFT4-GFP plasmid introduced into young leaves of 4-week-old tobacco (*Nicotiana tabacum*) plants by *Agrobacterium tumefaciens*, we found that the tomato 14-3-3 protein TFT4 was located in the nucleus, cytosol, and PM (Supplemental Fig. S3).

### $H^+$ Flux and PM $H^+$-ATPase Activity in Root Tips

To test whether PM $H^+$-ATPase-mediated $H^+$ secretion is involved in the *TFT4* response to alkaline stress, we analyzed PM $H^+$-ATPase activity and $H^+$ flux in the root tips (0–1,500 $\mu$m from the root cap junction [RCJ]) of *TFT4*-overexpressing Arabidopsis plants under control conditions (pH 5.8) and alkaline stress (pH 7.8) for 2 d (Fig. 3). Under alkaline stress, PM $H^+$-ATPase activities in OE-TFT4 (L4-1, L4-2, and L4-3) were significantly higher than those of the wild type. We examined changes in $H^+$ fluxes along the root tip of wild-type and *TFT4*-overexpressing plants, concentrating on the following zones: 50, 100, 150, 200, 250, 300, 400, 520, 600, 700, 850, 900, 1,000, 1,100, 1,200, and 1,500 $\mu$m from the RCJ under control conditions and following the imposition of alkaline stress. There was no significant difference in $H^+$ flux (influx and efflux) between wild-type and *TFT4*-overexpressing plants under control conditions. However, under alkaline stress, $H^+$ efflux in *TFT4*-overexpressing plants was significantly higher than that in wild-type plants in the elongation zone (520–850 $\mu$m from the RCJ) and the growth-termination zone (850–1,500 $\mu$m from the RCJ; $P < 0.05$).

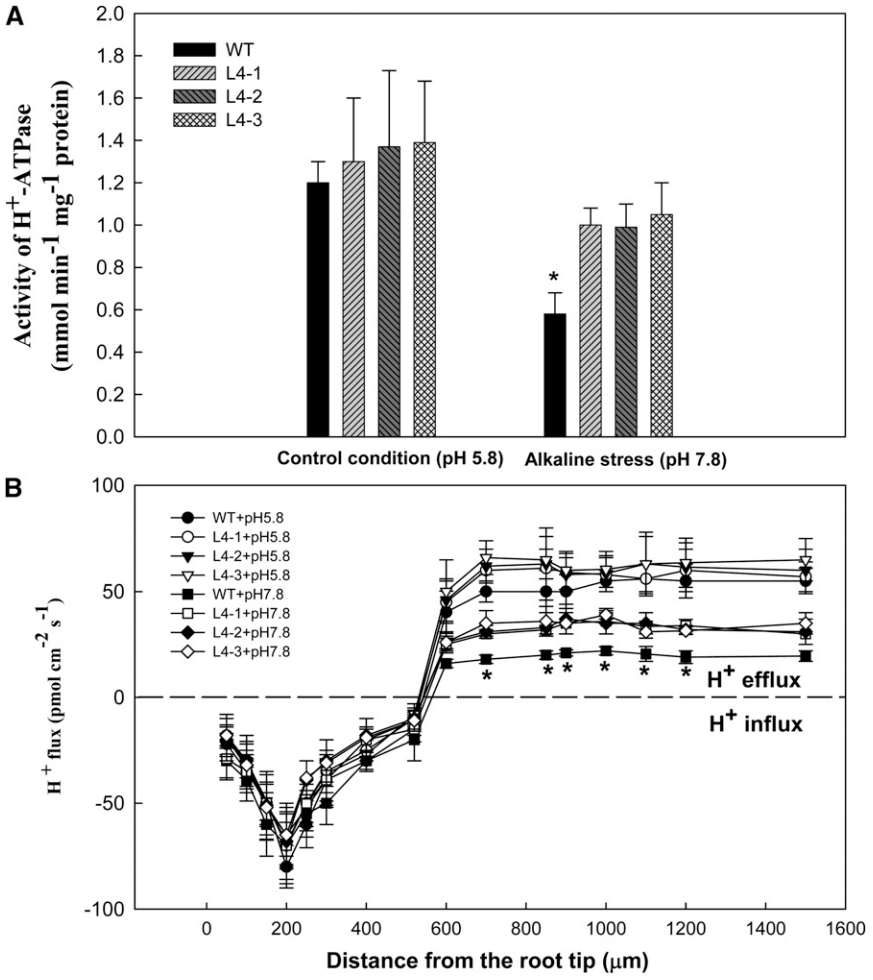


**Figure 3.** PM $H^+$-ATPase activity and $H^+$ flux of root tips (0–1,500 $\mu$m from the RCJ) in wild-type (WT; Col-0) and *TFT4*-overexpressing (L4-1, L4-2, and L4-3) Arabidopsis plants under control conditions (pH 5.8) and alkaline stress (pH 7.8) over 2 d. Asterisks represent significant differences compared with the wild type ($P > 0.05$).

### Performance of Grafted *TFT4*-Overexpressing Arabidopsis Plants under Alkaline Stress

To identify whether *TFT4* operates in the root or leaf under alkaline stress, we performed reciprocal grafting using wild-type and *TFT4*-overexpressing Arabidopsis plants (OE-TFT4: L4-2). Four combinations of grafted plants were generated in addition to two self-grafted controls: wild-type leaf to wild-type root (designated as WTL/WTR), L4-4 leaf to L4-2 root (OE-TFT4L/OE-TFT4R), and two reciprocal grafts, wild-type leaf to L4-2 root (WTL/OE-TFT4R) and L4-4 leaf to wild-type root (OE-TFT4L/WTR). Under control conditions (pH 5.8), no significant differences were observed in total root length, primary root elongation, or $H^+$ efflux among these grafting combinations (Fig. 4). However, under mild alkaline stress (pH 7.8), total root length, primary root elongation, and $H^+$ efflux in OE-TFT4L/OE-TFT4R and WTL/OE-TFT4R were significantly higher than those in WTL/WTR and OE-TFT4L/WTR. These results show that *TFT4* operates mainly in the root, regulating $H^+$ efflux.

### Auxin Transport in Wild-Type and *TFT4*-Overexpressing Arabidopsis Plants

To explore the molecular involvement of *TFT4* in the alkaline stress response, we used full genomic microarray hybridization to analyze the difference of the

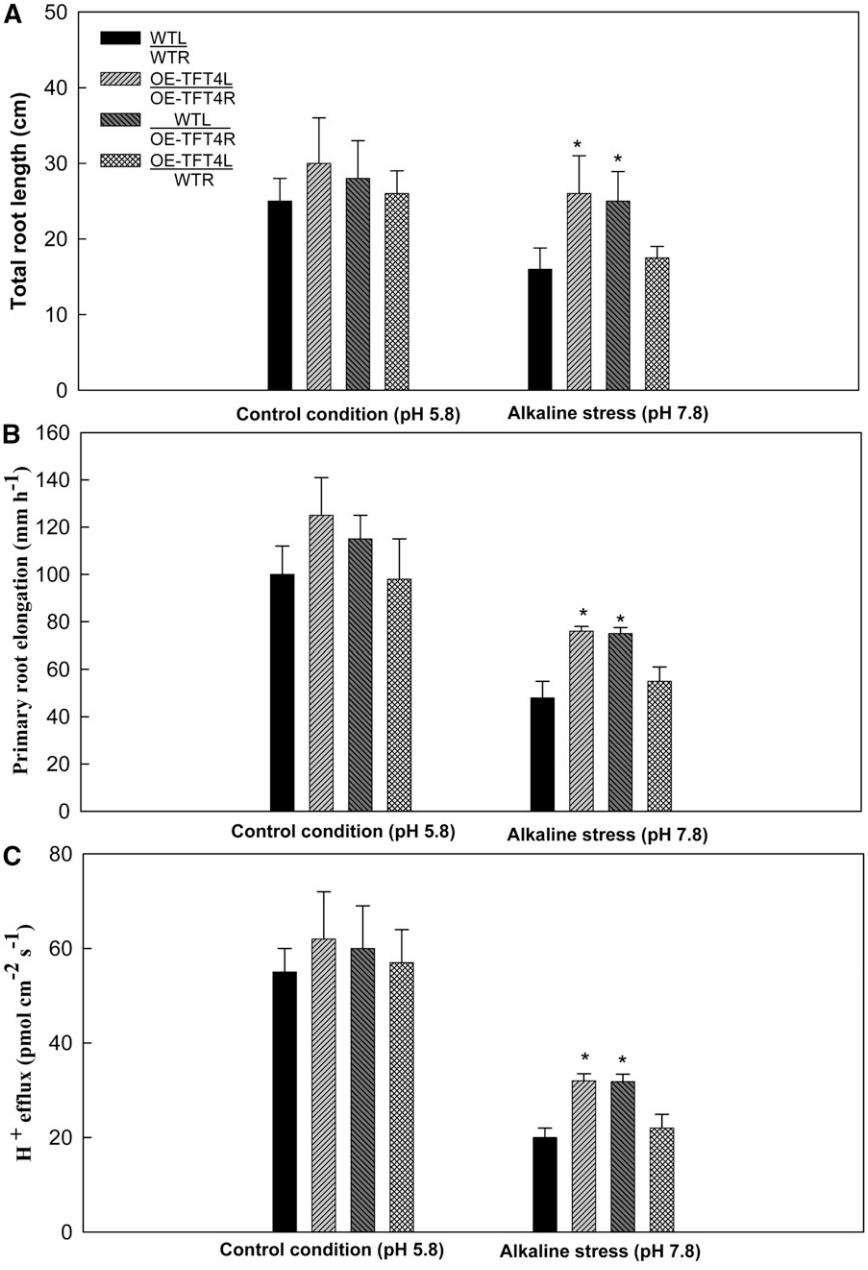


**Figure 4.** Total root lengths, elongation rates of primary roots, and $H^+$ efflux (750 $\mu$m from the RCJ) of plants resulting from grafts between wild-type (WTL and WTR for wild-type leaf and root, respectively; Col-0) and *TFT4*-overexpressing (L4-2) Arabidopsis plants under control conditions (pH 5.8) and alkaline stress (pH 7.8) over 2 d. Asterisks represent significant differences compared with self-grafted plants ($P > 0.05$).

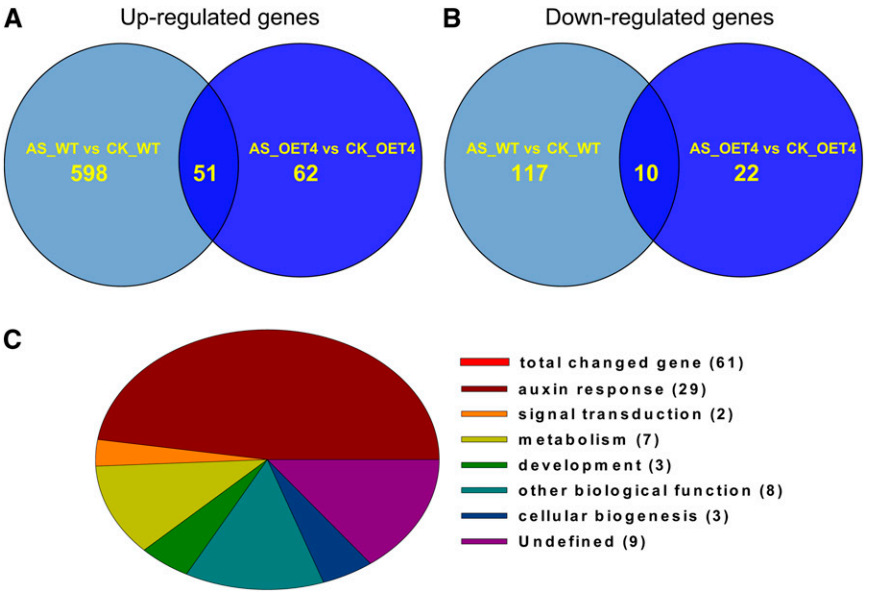


**Figure 5.** Identification of genes responding to alkaline stress (AS; pH 7.8 for 2 d) in root tips (0–1,500 $\mu$m from the RCJ) of *TFT4*-overexpressing Arabidopsis plants (L4-2) using microarray analysis. CK, Control condition (pH 5.8 for 2d); WT, wild type. [See online article for color version of this figure.]

transcriptome between wild-type and *TFT4*-overexpressing Arabidopsis plants. Microarray analysis (Affymetrix GeneChip) identified (in a comparison with the wild type) 61 alkaline stress-responsive genes in the root tip of OE-TFT4 (Fig. 5). Among these, 29 (about 48%) were IAA related (Supplemental Table S3). Real-time RT-PCR (Supplemental Fig. S4) demonstrated that the degree of up-regulated changes in *AUX1* (an IAA influx transport protein) and *PIN2* (an IAA efflux transport protein) under alkaline stress were closely concordant with microarray changes. Moreover, although no significant difference in IAA content was noted between wild-type and OE-TFT4 plants under control and alkaline stress conditions, IAA transport (basipetal direction) in OE-TFT4 was significantly higher than in the wild type under alkaline stress (Fig. 6). In addition, primary root elongation, basipetal IAA transport, and $H^+$ efflux in the wild type were significantly reduced in comparison with OE-TFT4 but higher than in mutant plants (*aux1*, *pin2*, OE-TFT4/*aux1*, and OE-TFT4/*pin2*) under alkaline stress (Fig. 7).

### Different Responses of *TFT4* and *TFT7*

Our previous results (Xu et al., 2012b) demonstrated that *TFT7* directly functions in the root by enhancing $H^+$ secretion under low-phosphorus stress. Therefore, we asked whether *TFT7* is involved under alkaline stress. We found that although no significant differences were observed in root basipetal IAA transport, $H^+$ efflux in OE-TFT4 and OE-TFT7 was significantly

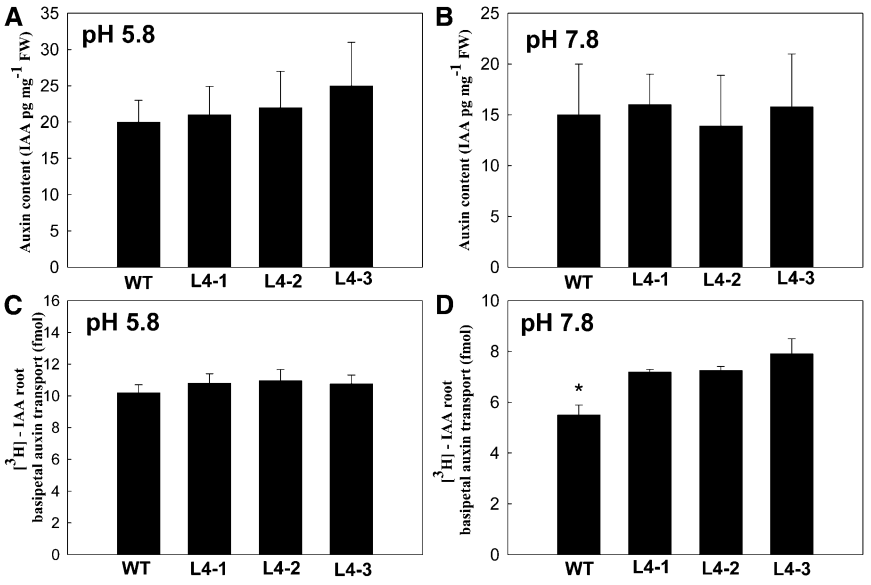


**Figure 6.** Endogenous auxin (IAA) contents and root basipetal auxin transport in the root tips of wild-type (WT; Col-0) and *TFT4*-overexpressing (L4-1, L4-2, and L4-3) Arabidopsis plants under control conditions (pH 5.8) and alkaline stress (pH 7.8) over 2 d. FW, Fresh weight.

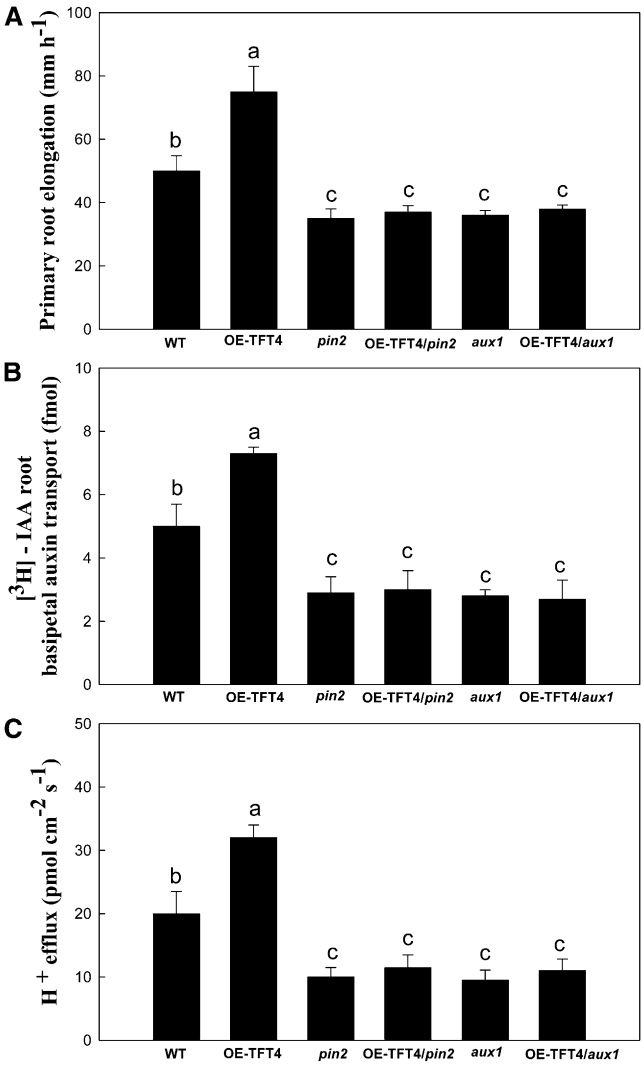


**Figure 7.** Elongation rates of primary roots, root basipetal auxin (IAA) transport, and $H^+$ efflux (750 $\mu$m from the RCJ) in wild-type (WT; Col-0), *TFT4*-overexpressing (OE-TFT4), and mutant (*aux1*, *pin2*, OE-TFT4/*aux1*, OE-TFT4/*pin2*) Arabidopsis plants under alkaline stress (pH 7.8) over 2 d.

higher than that in the wild type, OE-TFT1, and OE-TFT6 under low-phosphorus conditions (Supplemental Fig. S5). However, under alkaline stress, $H^+$ efflux and root basipetal IAA transport in OE-TFT4 were significantly higher than those in the wild type, OE-TFT1, OE-TFT6, and OE-TFT7 (Supplemental Fig. S5). In addition, in the wild type, OE-TFT1, OE-TFT6, and OE-TFT7, root basipetal IAA transport under alkaline stress was significantly lower than that under low-phosphorus conditions ($P < 0.05$).

In further experiments, root-tip responses of Arabidopsis plants were investigated under alkaline stress using auxin (IAA) treatments (exogenous auxin addition) and $H^+$ response regulation treatments (vanadate [VA], a PM ATPase inhibitor, and fusicoccin [FC], a PM ATPase enhancer). Figure 8 shows that in comparison with the simple alkaline stress treatment, VA addition did not alter root basipetal IAA transport (Fig. 8B) but significantly reduced primary root elongation (Fig. 8A) and root $H^+$ efflux (Fig. 8C) to the same degree among different Arabidopsis lines (the wild type, OE-TFT4, and OE-TFT7). In the combination treatment of alkaline stress plus FC, no significant differences were noted in these root-tip responses between the wild type and OE-TFT7. However, FC addition significantly improved primary root elongation and root $H^+$ efflux in OE-TFT4. In the combination treatment of alkaline stress plus IAA, root basipetal IAA transport was improved (in the wild type, OE-TFT4, and OE-TFT7); furthermore, primary root elongation and root $H^+$ efflux in OE-TFT4 and OE-TFT7 were significantly higher than in the wild type. In the combination treatment of alkaline stress plus IAA and VA, even though root basipetal IAA transport improved significantly (in the wild type, OE-TFT4, and OE-TFT7), primary root elongation and root $H^+$ efflux were lower than under simple alkaline stress. In addition, no significant differences were observed in these root-tip responses among lines (the wild type, OE-TFT4, and OE-TFT7) under the combination treatment of alkaline stress plus IAA and VA. Compared with the simple alkaline stress treatment, primary root elongation, root basipetal auxin transport, and root $H^+$ efflux were improved significantly (in the wild type, OE-TFT4, and OE-TFT7) in the combination treatment of alkaline stress plus IAA and FC.

Protein kinase PKS5 and chaperone J3 are known regulators of PM $H^+$-ATPase. We found that under alkaline stress, the expression of *PKS5* decreased in OE-TFT4, but the expression of *J3* was elevated (in comparison with the wild type and OE-TFT7; Supplemental Fig. S6). In addition, under alkaline stress, primary root elongation, root basipetal IAA transport, and root $H^+$ efflux of OE-TFT4/*pks5* were higher than those in *pks5* and OE-TFT4/*j3*. Root-tip responses of OE-TFT4/*j3* were higher than those of *j3* (Fig. 9) under alkaline stress. This suggests that TFT4 participates in the PKS5-J3 pathway in the enhancement of PM $H^+$-ATPase under alkaline stress.

## DISCUSSION

### *TFT4* Enhances $H^+$ Efflux and Basipetal Auxin Transport in the Root Tip

Previous studies have demonstrated that alkaline stress can inhibit plant growth more strongly than salt stress (Zhu, 2001; Yang et al., 2008; Guo et al., 2010), but few attempts have been made to investigate the molecular events involved. In general, root tips of higher plants display high sensitivity to environmental stimuli (Baluška and Mancuso, 2013) and might play a pivotal role in responses to alkaline stress. We previously showed that PIN2, an IAA efflux transporter, is involved in the acclimation and adaptation of Arabidopsis roots to alkaline stress by modulating $H^+$ secretion (Xu et al., 2012a), indicating that $H^+$ exudation and/or

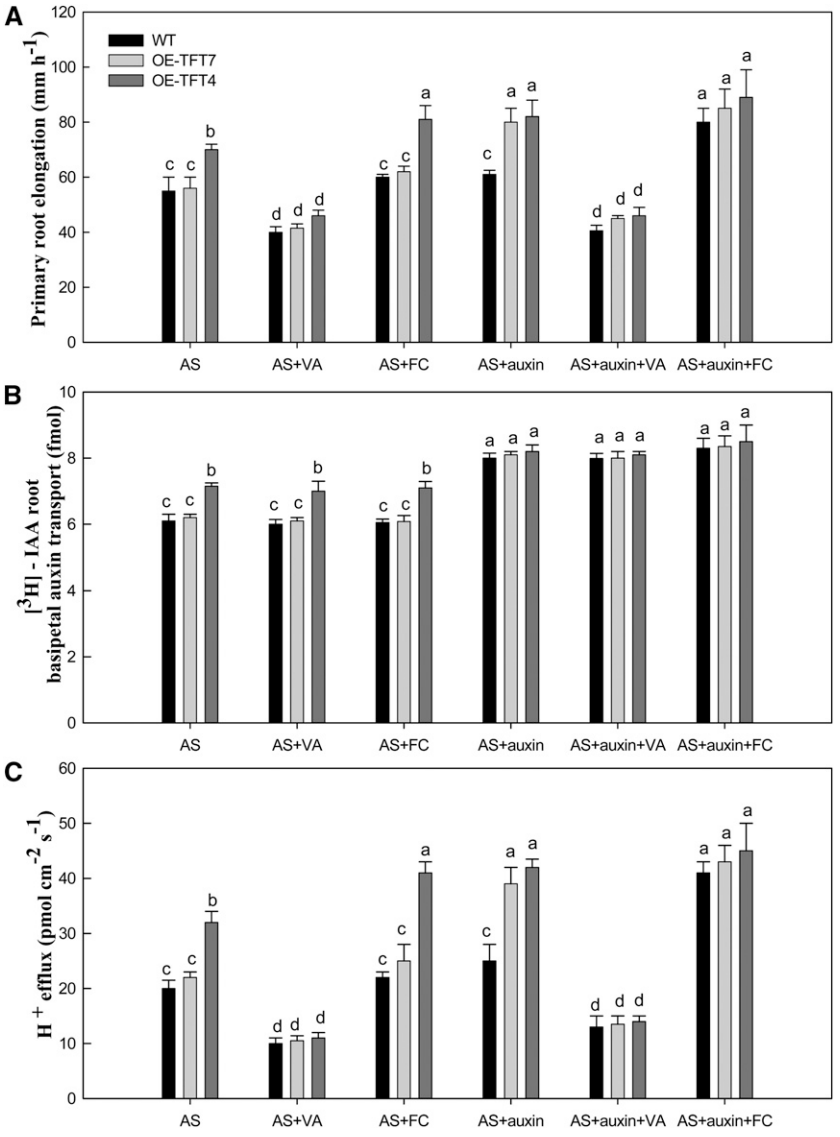


**Figure 8.** Root-tip responses of wild-type (WT; Col-0), *TFT4*-overexpressing (OE-TFT4), and *TFT7*-overexpressing (OE-TFT7) Arabidopsis plants under alkaline stress, auxin (IAA) treatment, and $H^+$ flux modification. Arabidopsis plants were subjected to alkaline stress (pH 7.8), VA (a PM ATPase inhibitor; 1 mM), FC (a PM ATPase stimulator; 10 $\mu$M), and exogenous auxin (IAA; 10 $\mu$M) over 2 d in hydroponic culture.

IAA transport play roles in root responses to alkaline stress. In this study, we found that *TFT4* operates predominantly in the root tip under alkaline stress by enhancing both $H^+$ efflux and IAA transport (Figs. 1–7; Supplemental Table S3; Supplemental Fig. S4). Although TFT4 gene expression in leaves was increased (Fig. 1), shoot dry weight of transgenic plants remained unaffected (Fig. 2). Our previous work shows that 14-3-3 proteins are involved in systemic responses of whole plants, such as to low-phosphorus stress (Xu et al., 2012b). Here, our grafting results suggest that TFT4 operates mainly in roots by regulating $H^+$ efflux-mediated root elongation under alkaline stress (Fig. 4). In addition, our results (Figs. 5–7) suggest that TFT4 modulates basipetal IAA transport in root tips. Thus, the increased expression of TFT4 in leaves (Fig. 1) might contribute to its operation in roots by becoming involved in the systemic response of the whole plant in response to alkaline stress while not necessarily affecting local organ growth, such as shoot growth (Fig. 2).

### *TFT1*, *TFT4*, *TFT6*, and *TFT7*, Four Tomato 14-3-3 Proteins, Have Distinct Actions under Alkaline Stress

14-3-3 proteins have a highly conserved target-binding domain able to recognize several short consensus amino acid motifs containing phosphoserine or phosphothreonine (Roberts and Bowles, 1999). Evidence is accruing that supports the notion that individual 14-3-3 isoforms have specific functions in higher plants (Sehnke et al., 2002; Xu et al., 2012b), although little genetic evidence currently exists for isoform specificity of plant 14-3-3 proteins under abiotic stress. We found that alkaline stress enhanced the gene expression of *TFT1*, *TFT4*, *TFT6*, and *TFT7* in tomato but that only *TFT4*-overexpressing Arabidopsis plants were tolerant of alkaline

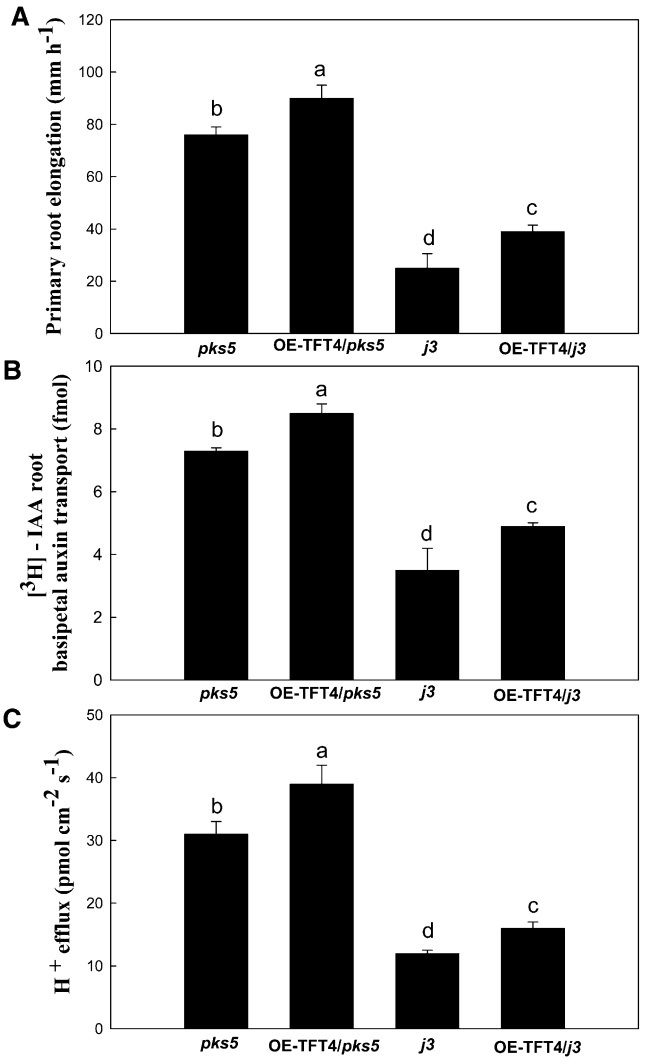


**Figure 9.** Primary root elongation, root basipetal auxin (IAA) transport, and root $H^+$ efflux of Arabidopsis plants (*pks5*, *j3*, OE-TFT4/*pks5*, and OE-TFT4/*j3*) under alkaline stress (pH 7.8) over 2 d.

stress (Fig. 2; Supplemental Fig. S2). We demonstrated previously that tomato 14-3-3 proteins respond to phosphorus deficiency (Xu et al., 2012b). Under low-phosphorus stress, *TFT6* was shown to act mainly in leaves by regulating leaf carbon allocation, while *TFT7* functioned directly in the roots, where it increased $H^+$ secretion. In the context of alkaline stress, several reports have proposed that the regulation of root $H^+$ secretion and basipetal IAA transport play important roles (Fuglsang et al., 2007; Yang et al., 2010; Xu et al., 2012a). We found that *TFT4* functions in $H^+$ efflux during root-tip acclimation to both alkaline and low-phosphorus stresses (Supplemental Fig. S5) and is involved in basipetal IAA transport under alkaline (Fig. 8), but not low-phosphorus, stress. *TFT7* improves root-tip $H^+$ efflux under low-phosphorus stress but does not act in basipetal auxin transport under either alkaline stress (Fig. 8) or low-phosphorus stress. It is instructive to ask why such different responses of *TFT4* and *TFT7* to alkaline and low-phosphorus stress exist in the regulation of IAA transport. According to the chemiosmotic model of polar IAA transport (Estelle, 1998), IAA is transported from cell to cell directionally. In the acidic cell wall environment of root cells, IAA can be transferred to the cytosol, together with a protonated form (IAAH), by AUX1-modulated IAA influx, which is maintained by PM ATPase-mediated $H^+$ efflux. In the nearly pH-neutral cytosol of the root cell, IAA loses a $H^+$ and transitions into the charged $IAA^-$ form and, as such, cannot diffuse out of the cell. However, $IAA^-$ can be moved outward via PIN2-medulated IAA efflux. Under low-phosphorus stress, the ratio of charged to noncharged state (IAAH versus $IAA^-$) might not be greatly affected. Under such conditions, both *TFT4* and *TFT7* are able to maintain basipetal IAA transport in root tips (Supplemental Fig. S5) and are then able to promote PM ATPase-mediated $H^+$ efflux. However, alkaline stress is expected to affect the ratio of charged to noncharged state (IAAH versus $IAA^-$) on account of the increasing extracellular pH. When this occurs, only *TFT4*, perhaps on account of its own pH optimum or pH-related signaling, can promote basipetal IAA transport in root tips (Supplemental Fig. S5), thus maintaining PM ATPase-mediated $H^+$ efflux.

### *TFT4* Integrates $H^+$ Efflux, Basipetal Auxin Transport, and the PKS5-J3 Pathway in Root Tips

Our previous work indicated that transported IAA promotes $H^+$ efflux by activating the PM ATPase in root tips, thus maintaining primary root elongation under water stress (Xu et al., 2013). Thus, we explored the modulation, by *TFT4*, of primary root elongation, $H^+$ efflux, and IAA transport under alkaline stress (Figs. 7 and 8). Compared with OE-TFT7 and the wild type, FC (a PM ATPase enhancer) can promote root elongation in OE-TFT4 due to the fact that *TFT4* is synchronously involved in the maintenance of $H^+$ efflux and IAA transport. Moreover, IAA addition can promote root elongation in OE-TFT4 and OE-TFT7 compared with the wild type due to the fact that *TFT4* and *TFT7* can maintain $H^+$ efflux. Additionally, when $H^+$ efflux is inhibited, IAA addition cannot affect the recovery of root elongation in OE-TFT4 and OE-TFT7. Furthermore, when IAA transport and $H^+$ efflux are concurrently maintained in root tips (IAA + FC), all three plant lines (the wild type, OE-TFT4, and OE-TFT7) are affected in primary root elongation under alkaline stress. Therefore, the synchronous modulation of $H^+$ efflux and auxin transport is important for the plant response to alkaline stress, and *TFT4* plays a pivotal role in this process.

Protein kinase PKS5 (as a negative regulator) and chaperone J3 (as a positive regulator) are required for root acclimation and adaptation to alkaline stress through their roles in regulating the interaction between 14-3-3 proteins and PM ATPase (Fuglsang et al., 2007; Yang et al., 2010). Accordingly, we investigated the gene expression of *PKS5* and *J3* in *TFT4*-overexpressing and

*TFT7*-overexpressing Arabidopsis plants under alkaline stress (Supplemental Fig. S6) and demonstrated that, while *TFT4* may be involved in the pathways of *PKS5* and *J3*, *TFT7* is not. Furthermore, when *PKS5* was deficient in *TFT4*-overexpressing Arabidopsis plants, *TFT4* improved its effects on primary root elongation, $H^+$ efflux, and IAA transport (Fig. 9). Similarly, when *TFT4* was overexpressed in the *j3* mutant, *TFT4* improved its effect on primary root elongation, $H^+$ efflux, and IAA transport. Therefore, *TFT4* emerges as an important regulator in PKS5-J3 pathways.

In summary, *TFT4* operates as an important modulator of $H^+$ efflux, auxin transport, and PKS5-J3 pathways in the response to alkaline stress in root tips. Elongating root cells are characterized by their ability to undergo cell wall extension under acidic apoplastic conditions. Thus, active $H^+$ efflux at the root tip plays a fundamental role in responses to alkaline stress by maintaining elongation of the primary root (Hager et al., 1991; Xu et al., 2012a), and *TFT4* promotes $H^+$ efflux at the root apex under alkaline stress and is an efficient regulator in the $H^+$-responsive pathway. Furthermore, IAA signaling and IAA cell-cell transport are key regulators of the acidity-induced cell elongation of the root (Hager, 2003; Xu et al., 2013). We also found that, under alkaline stress, the $H^+$ efflux response alone is insufficient and that IAA transport plays an essential supporting role in this process. For example, *TFT4* maintained elongation of the primary root by concomitantly promoting $H^+$ efflux and IAA transport in the root tip under alkaline stress. Although *TFT7* did not maintain elongation of the primary root under alkaline stress, exogenous IAA addition promoted the involvement of *TFT7* in IAA transport of the root tip. Following this exogenous application treatment, *TFT7* assisted in the response to alkaline stress by enhancing $H^+$ efflux (Fig. 8).

It is known that PKS5 inhibits $H^+$ efflux in root tips by preventing PM $H^+$-ATPase interaction with 14-3-3 proteins, and *J3* activates PM $H^+$-ATPase through the inactivation of PKS5 (Fuglsang et al., 2007; Yang et al., 2010). Our results indicate that *TFT4* plays a regulatory role in PKS5-J3 pathways under alkaline stress. Therefore, we propose the existence of an integrated modulation of $H^+$ efflux, IAA transport, and PKS5-J3 pathways. Under alkaline stress, the $H^+$ efflux response may constitute the initial response in the root tip. Subsequently, efficient IAA transport modulates PM $H^+$-ATPase activity to maintain $H^+$ efflux in the root tip. At the same time, PKS5 and J3 play important roles in the response to alkaline stress in root tips by regulating PM $H^+$-ATPase-mediated $H^+$ efflux. All three pathways integrate root apex responses to alkaline stress for the maintenance of primary root elongation, and *TFT4* plays pivotal roles in this process. However, it is important to point out that the demonstration of *TFT4* function here occurs in the Arabidopsis experimental system, and comprehensive analyses, including reverse genetics, reductions in the expression of the *TFT4* gene in tomato plants, and proteomic profiling, will be beneficial in the future to define the role and regulation of *TFT4* in the native species itself.

## MATERIALS AND METHODS

### Plant Growth Conditions and Stress Treatment

Tomato plants (*Solanum lycopersicum* 'Hezuo903') were grown hydroponically in black pots containing modified Hoagland solution with the following nutrients: $KNO_3$, 0.5 mM; $Ca(NO_3)_2$, 1.0 mM; $KH_2PO_4$, 1.0 mM; $MgSO_4$, 0.3 mM; $H_3BO_3$, 13.3 $\mu$M; $MnCl_2$, 3.0 $\mu$M; $CuSO_4$, 0.5 $\mu$M; $ZnSO_4$, 1.0 $\mu$M; $Na_2MoO_4$, 0.1 $\mu$M; NaCl, 2 $\mu$M; $CoCl_2$, 0.01 $\mu$M; $NiSO_4$, 0.1 $\mu$M; and Ethylenediamine-N,N′ -bis(2-hydroxyphenylacetic acid) Ferric sodium complex, 20 $\mu$M. The solution pH was adjusted to 5.8 (control condition) daily, and the solution was replaced every 2 d. To expose 12-d-old tomato plants to alkaline stress, we adjusted the solution pH to 7.5, 7.8, 8.0, or 8.2 every day using 0.1 mM KOH. Samples were harvested 0, 0.5, 1, 2, 3, and 5 d after first exposure of plants to control conditions and alkaline stress. The roots and leaves of tomato plants were then separated, frozen, and stored at –80°C. In addition, Arabidopsis (*Arabidopsis thaliana*) seeds were grown hydroponically in the sugar-free agar medium solution culture system used by Xu and Shi (2006). Twelve-day-old Arabidopsis plants were exposed to control conditions (pH 5.8) and several levels of alkaline stress (pH 7.5, 7.8, 8.0, or 8.2) for 0, 0.5, 1, 2, 3, and 5 d in a hydroponic system. Arabidopsis samples were frozen immediately in liquid nitrogen and stored at –80°C. Additionally, Arabidopsis plants (12 d old) were subjected to control conditions and alkaline stress in hydroponic medium, with or without the following additions: auxin (IAA), the PM ATPase inhibitor VA, and the PM ATPase stimulator FC. Each independent experiment was replicated three times; replicates contained 10 tomato or Arabidopsis plants. Samples from each replicate were harvested and analyzed separately. Furthermore, independent experiments were repeated twice, at different times, but with identical growth conditions.

### Obtaining Transgenic and Mutant Arabidopsis Plants

The full-length coding sequences of *TFT1* and *TFT4* were first amplified from the tomato complementary DNA library using PCR (Supplemental Table S1) and then ligated into the pMD18-T vector (TaKaRa). The vector was then digested using the *Bam*HI/*Sac*I double digestion process, and the resulting DNA was subcloned into pBI121 linearized by double digestion with the same restriction enzymes. The coding regions of *TFT1* and *TFT4* were confirmed by DNA sequencing. Transformation of wild-type Arabidopsis plants (Col-0) was achieved by the floral dip method (Clough and Bent, 1998). T3 homozygous transgenic lines (OE-TFT1: L1-1 to L1-6; OE-TFT4: L4-1 to L4-6) for kanamycin resistance were used subsequently for further studies (Supplemental Fig. S1). The wild-type Arabidopsis was ecotype Col-0, unless otherwise indicated. Some of the Arabidopsis plant material used in this study was described in our previous papers: OE-TFT6 (L6-1 to L6-6) and OE-TFT7 (L7-1 to L7-6; Xu et al., 2012b), the *pin2* mutant (*eir1-4*), the *aux1* mutant (*aux1-7*; Xu et al., 2013), and the *pks5* mutant (Xu et al., 2012a). The Arabidopsis *j3* mutant (Salk_132923) was obtained from the Arabidopsis Biological Resource Center; the homozygous *j3* mutant was identified by PCR using primers specific to the insertion transfer DNA. To generate doubly genetically modified Arabidopsis plants, mutants (*pin2*, *aux1*, *pks5*, and *j3*) were crossed with transgenic plants (OE-TFT4). Subsequently, homozygous doubly genetically modified plants were selected on the basis of specific primers and kanamycin resistance.

### Real-Time RT-PCR

Database searches were performed on the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and The Institute for Genomic Research (http://www.tigr.org) Web sites. Total RNA was extracted from tomato or Arabidopsis plants under control conditions (pH 5.8) and under alkaline stress (pH 7.5, 7.8, 8.0, or 8.2). Real-time RT-PCR was performed according to the method of Xu and Shi (2006). Gene-specific primers were designed using Primer 5 software (Supplemental Table S1). Because $\alpha$-tubulin is a strongly and constitutively expressed housekeeping gene in tomato plants (Wang et al., 2002), quantifications of the mRNA levels of tomato were based on comparisons with the level of $\alpha$-tubulin mRNA. Thus, $\alpha$-tubulin mRNA (standardized to 100 relative expression units) was used as

an internal standard in the gene expression of tomato plants. *AtACT2* is a strongly and constitutively expressed housekeeping gene in Arabidopsis (Panchuk et al., 2005). The quantification of Arabidopsis gene mRNA levels was based on comparisons with the level of mRNA for *AtACT2*, which was standardized to 100 relative expression units. The expression levels of a gene in tomato and Arabidopsis plants were defined by the ratio of the copy number of the gene studied to the copy number of $\alpha$-tubulin (tomato) or *AtACT2* (Arabidopsis) multiplied by 100 relative expression units. All transcripts of these studied genes were confirmed by DNA sequencing.

## Subcellular Localization of *TFT4*

The full-length coding sequence of *TFT4* was obtained by PCR (Supplemental Table S1) and cloned into the entry vector (pENTR TOPO vector) for the Gateway system (Invitrogen). After sequencing, the correct entry clone was recombined with the Gateway destination vector pGWB5 by using the LR reaction kit (Invitrogen). The correct p35S::TFT4-GFP plasmid formed was transformed into *Agrobacterium tumefaciens* GV3101 cells. The transformed *A. tumefaciens* cells were then used to infiltrate the young leaves of 4-week-old tobacco (*Nicotiana tabacum*) plants. About 40 h after infiltration, the expression of TFT4-GFP was analyzed by confocal microscopy (Olympus FV-1000 spectral type SPD mar/G/R IX81 FLUOVIEW laser confocal system), following the method of Xu et al. (2013). For GFP imaging, we used the 488-nm line for excitation, and emission was detected at 520 nm.

## Measurement of Plant Growth

To measure dry weight, we collected Arabidopsis plants, dried them at 70°C for 3 d, and then weighed them. Total root length and primary root length were measured using a root analysis instrument (WinRHIZO; Regent Instruments). The elongation rate of Arabidopsis primary roots ($\mu$m h$^{-1}$) was calculated by the spatial displacement of the primary root apex over unit time under control conditions (pH 5.8) and under alkaline stress (pH 7.5, 7.8, 8.0, or 8.2) following the method of Xu et al. (2012a).

## Assay of PM $H^+$-ATPase Activity and $H^+$ Flux along the Root Tip

PM $H^+$-ATPase activity at the root tip (0–1,500 $\mu$m from the RCJ) was determined following Xu et al. (2012a). $H^+$ fluxes were measured noninvasively using the scanning ion-selective electrode technique (SIET system BIO-003A; Younger USA Science and Technology; Applicable Electronics; Science Wares) following the method of Xu et al. (2012b). Arabidopsis $H^+$ fluxes were measured along the root tip, focusing on the following zones: 50, 100, 150, 200, 250, 300, 400, 520, 600, 700, 850, 900, 1,000, 1,100, 1,200, and 1,500 $\mu$m from the RCJ.

## Grafting of Arabidopsis Plants

We performed grafting of Arabidopsis plants grown on sterile vertical agar plates following Turnbull et al. (2002), with minor modifications. Seeds of wild-type and transgenic Arabidopsis plants were surface sterilized and stratified at 4°C for 3 d. The seeds were then spread on sterile one-half-strength Murashige and Skoog agar (0.8%) plates. The plates were held vertically under constant light (about 120 $\mu$mol photons m$^{-2}$ s$^{-1}$) at 21°C for 3 d in an Arabidopsis growth chamber and then maintained at 25°C under an 8-h photoperiod (60 $\mu$mol photons m$^{-2}$ s$^{-1}$) for another 3 d. Silicone tubing collars were used to hold grafts on seedlings, and plants subjected to this treatment were maintained on the same agar plates and under the same growth conditions for another 6 d until the graft junctions had healed. Successfully grafted Arabidopsis plants (12 d old) were treated under control conditions (pH 5.8) or alkaline stress (pH 7.8) under hydroponic conditions.

## Microarray Analysis

For Affymetrix GeneChip analysis, total RNA of Arabidopsis root tips (0–1,500 $\mu$m from the RCJ) was isolated using TRIzol reagent and purified with RNeasy spin columns (Qiagen). Five micrograms of total RNA was used to construct biotin-labeled complementary RNA products. We used the Affymetrix Arabidopsis ATH1 genome array GeneChip, which contains more than 22,500 probe sets representing approximately 20,000 genes. All the processes for complementary RNA preparation, labeling, hybridization, washing, and scanning were conducted following the GeneChip Standard Protocol (Affymetrix). GCOS (Affymetrix GeneChip operating software) was used for data collection and normalization. Differentially expressed genes were selected based on the following criteria: $\log_2$ (fold change) $> 1$ and $P < 0.05$.

## Assay of Auxin Content and Transport

We measured the auxin (IAA) content of Arabidopsis plants by gas chromatography-selected reaction monitoring-mass spectrometry, following Ljung et al. (2005). The root tips (0–1,500 $\mu$m from the RCJ) were collected, and six replicates of the samples were purified after the addition of 250 pg of [$^{13}C_6$] IAA internal standard. To analyze root basipetal auxin transport in Arabidopsis plants, we placed 1% (w/v) agar blocks containing 100 nM [$^3$H]IAA so that they were in contact with the root tips (0–1,500 $\mu$m from the RCJ) for 5 h in darkness. After that, treated root tips were excised for radioactivity counting following Xu et al. (2013).

## Statistical Analyses

Data were subjected to ANOVA and post hoc comparisons (Duncan's multiple range test; $P < 0.05$ significance level). We used SPSS version 13.0 software. Values presented are means ± SD of six replicates from two independent experiments. Shared lowercase letters a, b, or c attached to means in figures indicate no significant differences ($P > 0.05$) according to the post hoc tests.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1**. Expression of *TFT1* and *TFT4* (tomato 14-3-3 gene) in wild-type Arabidopsis plants (WT), *TFT1*-overexpressing Arabidopsis lines (L1-1 to L1-6), and *TFT4*-overexpressing Arabidopsis lines (L4-1 to L4-6) demonstrated by real-time RT-PCR.

**Supplemental Figure S2**. Dry weights of whole plants (measured over 5 d) of the wild type (Col-0), OE-TFT1 (L1-6), OE-TFT4 (L4-2), OE-TFT6 (L6-3), and OE-TFT7 (L7-5) under control conditions (pH 5.8) and under alkaline stress (pH 7.5, 7.8, 8.0, or 8.2).

**Supplemental Figure S3**. Subcellular localization of the tomato 14-3-3 protein TFT4 in the tobacco system.

**Supplemental Figure S4**. Expression of *AUX1* (an auxin influx transport protein) and *PIN2* (an auxin efflux transport protein) in root tips (0–1,500 $\mu$m from the RCJ) of wild-type Arabidopsis plants (WT) and a *TFT4*-overexpressing Arabidopsis line (L4-2) under alkaline stress (pH 7.8 over 5 d) demonstrated by real-time RT-PCR.

**Supplemental Figure S5**. $H^+$ efflux (750 $\mu$m from the RCJ) and root basipetal auxin (IAA) transport of the wild type (Col-0), OE-TFT1 (L1-6), OE-TFT4 (L4-2), OE-TFT6 (L6-3), and OE-TFT7 (L7-5) under alkaline stress (pH 7.8) and low phosphorus (2 $\mu$M) over 2 d.

**Supplemental Figure S6**. Expression of *PKS5* (a protein kinase) and *J3* (a chaperone) in root tips (0–1,500 $\mu$m from the RCJ) of wild-type Arabidopsis plants (WT), a *TFT4*-overexpressing Arabidopsis line (L4-2), and a *TFT7*-overexpressing Arabidopsis line (L7-5) under alkaline stress (pH 7.8 over 2 d) demonstrated by real-time RT-PCR.

**Supplemental Table S1**. Gene-specific primers used in real-time RT-PCR.

**Supplemental Table S2**. Expression of Arabidopsis endogenous 14-3-3 genes in wild-type and transgenic plants (TFT4-overexpressing lines L4-1 to L4-6) under control conditions (pH 5.8) and alkaline stress (pH 7.8) over 1 d.

**Supplemental Table S3**. Up- and down-regulation of auxin (IAA)-related genes in the root tips (0–1,500 $\mu$m from the RCJ) of OE-TFT4 Arabidopsis plants compared with the wild type under alkaline stress (pH 7.8) over 2 d.

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