SlKIX8 and SlKIX9 are conserved repressors of organ size in the asterid species tomato

Gwen Swinnen[1,2], Alexandra Baekelandt[1,2], Rebecca De Clercq[1,2], Jan Van Doorsselaere[3], Nathalie Gonzalez[4], Dirk Inzé[1,2], Alain Goossens[1,2,*] and Laurens Pauwels[1,2,*]

[1]Ghent University, Department of Plant Biotechnology and Bioinformatics, 9052 Ghent, Belgium

[2]VIB Center for Plant Systems Biology, 9052 Ghent, Belgium

[3]VIVES, 8800 Roeselare, Belgium

[4]INRAE, Université de Bordeaux, BFP, 33882 Villenave d'Ornon Cedex, France

ORCID IDs: 0000-0002-4438-0098 (G.S.); 0000-0003-0816-7115 (A.B.); 0000-0002-4068-1811 (R.D.C.); 0000-0002-3768-2434 (J.V.D.); 0000-0002-3946-1758 (N.G.), 0000-0002-3217-8407 (D.I.); 0000-0002-1599-551X (A.G.); 0000-0002-0221-9052 (L.P.)

Corresponding authors:

Laurens Pauwels

VIB-UGent Center for Plant Systems Biology

Technologiepark 71, B-9052 Gent (Belgium)

Tel.: +32 9 331 39 71 ; Fax: +32 9 331 38 09

E-mail: laurens.pauwels@psb.vib-ugent.be

Alain Goossens

VIB-UGent Center for Plant Systems Biology

Technologiepark 71, B-9052 Gent (Belgium)

Tel.: +32 9 331 38 51; Fax: +32 9 331 38 09

E-mail: alain.goossens@psb.vib-ugent.be

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Summary

Plant organ size and shape are major agronomic traits that depend on cell division and expansion, which are both regulated by complex gene networks. In several eudicot species belonging to the rosid clade, organ growth is controlled by a repressor complex consisting of PEAPOD (PPD) and KINASE-INDUCIBLE DOMAIN INTERACTING (KIX) proteins. Whether the function of these proteins as regulators of organ size is conserved in asterids as well, which together with the rosids constitute most of the core eudicot species, is still unknown. We used CRISPR-Cas9 genome editing to target *SlKIX8* and *SlKIX9* in the asterid model species tomato (*Solanum lycopersicum*) and analyzed loss-of-function phenotypes. We found that loss of function of *SlKIX8* and *SlKIX9* led to the production of enlarged, dome-shaped leaves and that these leaves exhibited increased expression of putative SlPPD target genes. Moreover, *kix8 kix9* mutants carried bigger fruits with increased pericarp thickness. Protein interaction assays also confirmed that SlKIX8 and SlKIX9 act as molecular bridges between the SlPPD and SlTOPLESS co-repressor proteins. Our results show that KIX8 and KIX9 are conserved regulators of organ size in distinct eudicot species and can thus provide strategies to improve yield in fruit crops.

Introduction

Plants come in all shapes and sizes, yet these agronomically important traits are remarkably uniform within a given species or variety. Not surprisingly, cell division and cell expansion, the underlying processes of organ development, are under tight genetic control (Gonzalez *et al.*, 2012; Hepworth & Lenhard, 2014; Kalve *et al.*, 2014; Vercruysse *et al.*, 2020). The different phases of leaf development, for instance, are regulated by complex gene networks (Gonzalez *et al.*, 2012; Hepworth & Lenhard, 2014). Leaf development consists of the emergence of a leaf primordium from the shoot apical meristem, followed by a period of primary cell division that transitions into a cell expansion phase, and a simultaneous phase of meristemoid division. In Arabidopsis (*Arabidopsis thaliana*) leaves, the shape of the primary cell cycle arrest front, which moves from tip to base as cells cease to divide, was reported to be regulated by the transcriptional regulator PEAPOD2 (AtPPD2) (Baekelandt *et al.*, 2018). Through its interaction with the adaptor protein NOVEL INTERACTOR OF JAZ (AtNINJA)

(Fig. S1A), AtPPD2 can recruit the transcriptional co-repressor TOPLESS (AtTPL) and, thereby, control leaf flatness (Baekelandt *et al.*, 2018). In addition, AtPPD2 forms a transcriptional repressor complex with KINASE-INDUCIBLE DOMAIN INTERACTING 8 (AtKIX8)/AtKIX9 (Fig. S1a) (Gonzalez *et al.*, 2015), which can also recruit AtTPL, to limit the number of self-renewing asymmetric divisions that stem-cell like meristemoids can undergo before differentiating into stomatal guard cells (White, 2006; Gonzalez *et al.*, 2015). This way, the AtPPD2-AtKIX8/AtKIX9 repressor complex restricts leaf growth, thereby controlling both leaf shape and size (White, 2006; Gonzalez *et al.*, 2015). The functionalities of AtKIX8 and AtKIX9 are, thus, required for the repressive activity of AtPPD2 (Gonzalez *et al.*, 2015). Consequently, double *kix8-kix9* Arabidopsis knockout plants display increased transcript levels of AtPPD2 target genes and enlarged, dome-shaped leaves because of a prolonged period of meristemoid division, similar to Arabidopsis *ami-ppd* plants (Gonzalez *et al.*, 2015).

In Arabidopsis, the AtKIX8 and AtKIX9 proteins are known to interact with both AtPPD1 and AtPPD2 through their distinguishing N-terminal PPD domain (Fig. S1a) (Bai *et al.*, 2011) (Gonzalez *et al.*, 2015). Together with AtTIFY8 and the JASMONATE ZIM DOMAIN (AtJAZ) proteins, AtPPD1 and AtPPD2 belong to class II of the TIFY protein family (Vanholme *et al.*, 2007; Bai *et al.*, 2011) and are characterized by the presence of a conserved TIF[F/Y]XG motif. This motif resides within the ZINC-FINGER PROTEIN EXPRESSED IN INFLORESCENCE MERISTEM (ZIM) domain that mediates the interaction of class II TIFY proteins with AtNINJA (Fig. S1a) (Chini *et al.*, 2009; Chung & Howe, 2009; Pauwels *et al.*, 2010; Baekelandt *et al.*, 2018). All class II proteins, except AtTIFY8, contain a C-terminal Jas domain (Vanholme *et al.*, 2007; Bai *et al.*, 2011). The Jas domain found in AtPPD proteins, however, is divergent from the Jas consensus motif in AtJAZ proteins (Bai *et al.*, 2011) that mediates the interaction of AtJAZ proteins with transcription factors such as AtMYC2 and the F-box protein CORONATINE INSENSITIVE 1 (AtCOI1) (Chini *et al.*, 2007; Thines *et al.*, 2007).

Next to AtKIX8 and AtKIX9, nine other proteins that contain a KIX domain have been described in Arabidopsis (Thakur *et al.*, 2013). Both in plant and non-plant species, such as yeast and humans, the KIX proteins include HISTONE ACETYLTRANFERASE (HAT) proteins and Mediator subunits (Kumar *et al.*, 2018; Thakur *et al.*, 2013) that are known to function as co-activators through the interaction

of their KIX domain with the transactivation domain of transcription factors (Thakur *et al.*, 2014; Kumar *et al.*, 2018). AtKIX8/AtKIX9 and their orthologs, however, are specific to plants and show, except for their N-terminal KIX domain, no similarity to these HAT and Mediator co-activators (Thakur *et al.*, 2013). Instead, they contain an ETHYLENE RESPONSE FACTOR (ERF)-ASSOCIATED AMPHIPHILIC REPRESSION (EAR) motif that allows them to recruit the co-repressor AtTPL (Kagale *et al.*, 2010; Causier *et al.*, 2012; Gonzalez *et al.*, 2015). Through their KIX domain, AtKIX8 and AtKIX9 can simultaneously interact with the transcriptional repressor AtPPD2 (Gonzalez *et al.*, 2015). Hence, AtKIX8/AtKIX9 forms a molecular bridge between AtPPD2 and AtTPL (Fig. S1a) and, because of that, AtPPD2 can act as a negative transcriptional regulator (Gonzalez *et al.*, 2015).

In Arabidopsis, the activity of the AtPPD-AtKIX repressor complex is regulated by the F-box protein STERILE APETALA (AtSAP) (Wang *et al.*, 2016; Li *et al.*, 2018). Interaction of the repressor complex with AtSAP results in the proteasomal degradation of both AtKIX and AtPPD proteins (Fig. S1a) (Wang *et al.*, 2016; Li *et al.*, 2018). In accordance with these observations, *AtSAP* overexpression plants produce enlarged rosettes composed of enlarged and dome-shaped leaves (Wang *et al.*, 2016).

Orthologs of the AtPPD, AtKIX, and AtSAP proteins were found in members of both eudicot and monocot species, but appear to be absent from Poaceae species (grasses), suggesting that the PPD-KIX-SAP module was lost in the grass lineage (Gonzalez *et al.*, 2015; Wang *et al.*, 2016). It has been suggested that this might reflect the absence of self-renewing meristemoids in the stomatal lineage of grasses (Liu *et al.*, 2009; Vatén & Bergmann, 2012; Gonzalez *et al.*, 2015; Wang *et al.*, 2016). Several eudicot members, in which orthologs of the *AtPPD* or *AtKIX* genes were mutated or downregulated, including *Medicago truncatula*, soybean (*Glycine max*), blackgram (*Vigna mungo*), and pea (*Pisum sativum*), produced enlarged leaves (Ge *et al.*, 2016; Naito *et al.*, 2017; Kanazashi *et al.*, 2018; Li *et al.*, 2019). Moreover, overexpression of *AtSAP* orthologs in *Medicago truncatula*, poplar (*Populus trichocarpa*) and cucumber (*Cucumis sativus* L.) increased leaf size as well (Yordanov *et al.*, 2017; Yang *et al.*, 2018; Yin *et al.*, 2020). Next to enlarged leaves, also increases in the size of other organs, such as stipules, flowers, fruits and seeds, were observed for several of said mutants (Ge *et al.*, 2016; Naito *et al.*, 2017; Kanazashi *et al.*, 2018; Yang *et al.*, 2018; Li *et al.*, 2019; Yin *et al.*, 2020). Control of organ growth by the PPD-KIX repressor complex, together with its post-translational regulation by the F-box protein SAP, thus,

seems to be conserved among distinct eudicot species. However, differential developmental stages might be targeted by the repressor complex depending on the species. In *Medicago truncatula* plants in which an *AtPPD* ortholog was mutated, for instance, a prolonged period of primary cell division was reported to be responsible for the increase in organ size (Ge *et al.*, 2016), whereas the enlarged leaf phenotype of Arabidopsis *kix8-kix9* and *ami-ppd* mutants was attributed to an extended duration of meristemoid division (Gonzalez *et al.*, 2015). The role of conserved regulators can thus vary considerably between different species, illustrating that the translation of knowledge on transcriptional regulators between species is not straightforward (Nelissen *et al.*, 2014; Kajala *et al.*, 2020). All of the aforementioned eudicot species, in which the function of the PPD-KIX-SAP module was described, belong to the rosid clade. Together with the asterids, the rosids constitute most of the core eudicot species (Fig. S1b) and their most recent common ancestor existed over 100 million years ago (Wikström *et al.*, 2001). Whether the orthologs of PPD, KIX, and SAP proteins function as regulators of organ growth in asterid members is still unknown.

Here, we report a conserved role for SlKIX8 and SlKIX9 in the regulation of organ growth in the asterid model species tomato (*Solanum lycopersicum*). We used protein interaction assays in yeast to demonstrate that the tomato orthologs of AtKIX8 and AtKIX9 function as SlTPL adaptor proteins for the SlPPD proteins. Next, we used CRISPR-Cas9 genome editing to simultaneously knockout *SlKIX8* and *SlKIX9* in the cultivar Micro-Tom. Double *kix8 kix9* tomato knockout lines produced enlarged, dome-shaped leaves and displayed increased expression of genes orthologous to AtPPD2 target genes. Finally, we revealed that these *kix8 kix9* tomato mutants carried larger fruits with increased pericarp thickness, both important agronomic traits for fruit crops.

Results

SlKIX8 and SlKIX9 are SlTPL adaptors for SlPPD proteins

To identify the tomato orthologs of the AtKIX8 and AtKIX9 proteins, BLASTP was used. The tomato orthologs of the AtPPD proteins have been described previously (Chini *et al.*, 2017). The SlKIX and SlPPD proteins display a similar domain structure as their Arabidopsis counterparts (Fig. S2). Amplification of the coding sequences of *SlKIX* and *SlPPD* genes revealed alternative splicing for *SlKIX9*, *SlPPD1*, and *SlPPD2* (Fig. S2b,c,d). Based on an alternative splicing model for *AtKIX9* reported by The

Arabidopsis Information Resource (TAIR), we hypothesized that retention of the second *SlKIX9* intron leads to the use of a downstream start codon, generating a splice variant that lacks the N-terminal KIX domain (Fig. S2b). The splice variants of *SlPPD1* and *SlPPD2* display retention of the Jas intron and part of the Jas intron, respectively, which is located between the two exons encoding the Jas domain (Fig. S2c,d). These alternative splicing events are proposed to generate premature stop codons (Fig. S2c,d), and consequently truncated SlPPD proteins, as was previously shown for *AtPPD1* and *AtPPD2* (Li *et al.*, 2016).

Previously, the interactions between KIX and PPD proteins from Arabidopsis and pea were analyzed *in vivo* and in detail in yeast (Gonzalez *et al.*, 2015; Li *et al.*, 2019). To determine whether SlKIX8, SlKIX9, SlPPD1, and SlPPD2 are part of a similar protein complex, we performed yeast two-hybrid (Y2H) assays. For these assays, the proteins encoded by the first splice variants shown in Fig. S2 were used. In case of the SlKIX proteins, these possessed the KIX domain, which was shown to be essential for mediating the interaction between KIX and PPD proteins from Arabidopsis and pea (Gonzalez *et al.*, 2015; Li *et al.*, 2019). Direct interaction between the SlKIX and SlPPD proteins could be observed (Fig. 1a). Next, we evaluated whether the SlKIX proteins were able to interact with SlTPL1 (Fig. 1b), which is the most closely related tomato ortholog of the Arabidopsis co-repressor AtTPL (Hao *et al.*, 2014). As only SlKIX8 was capable of interacting with SlTPL1 in the Y2H assays (Fig. 1b), we also assessed the interaction between the SlKIX proteins and the five additional SlTPL proteins that were reported in tomato (Fig. 1b). In addition to SlTPL1, SlKIX8 also interacted with SlTPL2, SlTPL4, and SlTPL6, whereas SlKIX9 could solely interact with SlTPL2 (Fig. 1b). By means of yeast three-hybrid (Y3H) assays, we subsequently demonstrated that the SlKIX proteins can form a molecular bridge between these SlTPL proteins and the SlPPD proteins (Fig. 1c,d). In Arabidopsis, both AtKIX8 and AtKIX9 were reported to interact with the F-box protein AtSAP, resulting in their post-translational degradation (Li *et al.*, 2018). However, we only observed interaction between SlKIX8 and the tomato ortholog of AtSAP (Fig. 1e). Taken together, our results suggest that in tomato, SlKIX8 and SlKIX9 function as SlTPL adaptors for the SlPPD proteins, similar to their orthologs in Arabidopsis.

CRISPR-Cas9 genome editing of *SlKIX8* and *SlKIX9* leads to enlarged dome-shaped leaves

To investigate the *in planta* role of SlKIX8 and SlKIX9, double *kix8 kix9* loss-of-function mutants (cultivar Micro-Tom) were generated using Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9 (CRISPR-Cas9) genome editing (Fig. 2a). A rippled, dome-shaped leaf phenotype could already be observed in regenerated double *kix8 kix9* tomato knockout (T0) plants (Fig. S3). Likewise, the progeny of two independent T1 plants mono- or biallelic for out-of-frame mutations at both the *SlKIX8* and *SlKIX9* loci (Fig. S4a,b), displayed dome-shaped leaves with uneven leaf laminae (Fig. 2b,c). Single *kix8* mutants (Fig. S4a,b), obtained by pollinating the *kix8 kix9*$^{\#1}$ (T1) line with wild-type pollen, exhibited an intermediate leaf phenotype (Fig. 2b,c), whereas single *kix9* plants (Fig. S4a,b) did not show any visible phenotype (Fig. 2b,c) as was noted for Arabidopsis *kix8* and *kix9* single mutants (Gonzalez *et al.*, 2015). These observations suggest that SlKIX8 and SlKIX9 may have partially redundant roles in regulating tomato leaf growth.

Given that the leaf shape phenotype was most pronounced for double *kix8 kix9* tomato mutants, phenotypical analyses were performed on leaf eight and compared with those on the corresponding wild-type leaf. First, leaf fresh weight was determined, which was approximately 30% higher for *kix8 kix9* leaves compared with wild-type leaves (Fig. 3a). Likewise, the fresh weight of the terminal leaflets of these *kix8 kix9* leaves was increased with approximately 40% compared with those of wild-type leaves (Fig. 3b). Next, the area of terminal leaflets was measured before (projected area) and after (real area) terminal leaflets were cut to flatten them (Fig. 3c,d) (Baekelandt *et al.*, 2018). After flattening the terminal leaflets, those of *kix8 kix9* mutants displayed an area that was approximately 40% larger than corresponding wild-type leaflets (Fig. 3e). In addition, the decrease in projected-to-real terminal leaflet area was about two times bigger for *kix8 kix9* plants compared with wild-type plants (Fig. 3f), demonstrating the alteration in *kix8 kix9* leaflet shape. These measurements, thus, substantiate the enlarged, dome-shaped leaf phenotype of double *kix8 kix9* tomato knockout plants.

Orthologs of AtPPD2 target genes are upregulated in leaves of tomato *kix8 kix9* mutants

To gain further insight into the function of SlKIX8 and SlKIX9 in tomato plants, we made use of public transcriptome data (cultivar Micro-Tom) (Zouine *et al.*, 2017) to investigate the gene expression patterns of *SlKIX8*, *SlKIX9*, *SlPPD1*, and *SlPPD2* in different tissues and throughout distinct developmental stages. A survey of these publicly available transcriptome data revealed that *SlKIX8* was low expressed in all examined tissues and that *SlKIX9* expression was (almost) absent in most tissues (Fig. 4a and Table S1). In all investigated tissues, the transcript level of *SlPPD2* was higher than that of *SlPPD1* (Fig. 4a and Table S1). Next, we looked up the gene expression patterns of the putative tomato orthologs of Arabidopsis *DWARF IN LIGHT 1* (*AtDFL1*), *AT-HOOK MOTIF CONTAINING NUCLEAR LOCALISED PROTEIN 17* (*AtAHL17*), and *SCHLAFMUTZE* (*AtSMZ*), which were top-ranked in the list of differentially expressed genes in Arabidopsis *ami-ppd* leaves and strongly upregulated in Arabidopsis *kix8-kix9* leaves (Gonzalez *et al.*, 2015). Expression of all three tomato genes, *SlDFL1*, *SlAHL17*, and *APETALA 2d* (*SlAP2d*), was confirmed in tomato leaves (Fig. 4b and Table S1). To verify the potential differential expression of these genes in tomato *kix8 kix9* mutants compared with wild-type plants, we performed a quantitative real-time PCR (qPCR) analysis on the terminal leaflet of growing leaves and found that the transcription of all three genes was upregulated in *kix8 kix9* mutants (Fig. 4c). Furthermore, the expression of *SlKIX8* and *SlKIX9* was increased in *kix8 kix9* plants compared with wild-type plants (Fig. 4d), suggesting negative feedback of the SlPPD-SlKIX complex on the expression of *SlKIX8* and *SlKIX9*. These findings indicate that SlKIX8 and SlKIX9 are required for the repression of tomato genes orthologous to three AtPPD2 target genes (*AtDFL1*, *AtAHL17*, and *AtSMZ*).

Tomato *kix8 kix9* mutants produce enlarged fruits

In multiple eudicot species that belong to the rosid order of Fabales, orthologs of the KIX and PPD proteins have been reported to negatively regulate seed pod size (Ge *et al.*, 2016; Naito *et al.*, 2017; Kanazashi *et al.*, 2018; Li *et al.*, 2019). Moreover, the cucumber ortholog of the F-box protein AtSAP was shown to positively regulate cucumber fruit size (Yang *et al.*, 2018). To examine whether the SlKIX proteins might also have a role in determining tomato fruit size in the asterid model species tomato,

we investigated whether the development of reproductive organs was affected in tomato *kix8 kix9* mutants. When growing the *kix8 kix9* mutants, we noted a significant delay in flowering time compared with wild-type plants (Fig. 5a). In addition, though seed size and number were not altered (Fig. S5a,b), we did observe an increase in *kix8 kix9* fruit size compared with wild-type fruits (Fig. 5b). The fresh weight of tomatoes produced by *kix8 kix9* plants was increased with approximately 80% compared with those produced by wild-type plants (Fig. 5c). Accordingly, the diameter of *kix8 kix9* fruits was approximately 20% larger than that of wild-type fruits (Fig. 5d). Cutting along the equatorial plane revealed that fruits from *kix8 kix9* mutants displayed an approximate increase of 70% in pericarp thickness compared with wild-type fruits (Fig. 6a,b). Altogether, these data demonstrate that knocking out *SlKIX8* and *SlKIX9* in tomato results in the production of enlarged fruits, a favorable agronomic trait, suggesting that SlKIX8 and/or SlKIX9 are involved in the regulation of tomato fruit growth.

Discussion

KIX8 and KIX9 are conserved regulators of leaf growth in distinct eudicot species

In Arabidopsis, the asymmetric cell division of meristemoids, which are precursor cells of the stomatal lineage, positively affects final leaf size and is restricted by a transcriptional repressor complex in which the co-repressor AtTPL is recruited to AtPPD2 by AtKIX8/AtKIX9 (White, 2006; Gonzalez *et al.*, 2015). Members of this repressor complex were shown to regulate leaf size and shape in a variety of species that belong to different orders of the rosids (Gonzalez *et al.*, 2015; Ge *et al.*, 2016; Naito *et al.*, 2017; Kanazashi *et al.*, 2018; Li *et al.*, 2019), suggesting that the repressor complex is a conserved regulator of leaf growth among rosid eudicots. Here, we demonstrate that the tomato orthologs of AtKIX8 and AtKIX9 act as SlTPL adaptors for SlPPD proteins and, thereby, regulate leaf growth in tomato plants. Tomato is a model species of the asterid clade that also includes tobacco (*Nicotiana tabacum*), carrot (*Daucus carota*) and sunflower (*Helianthus annuus*). In the rosid species Arabidopsis and pea, the interaction between KIX and PPD proteins is described to occur through the N-terminal KIX and PPD domain, respectively (Gonzalez *et al.*, 2015; Li *et al.*, 2019). This is likely to be the case in tomato as well, in which the SlKIX and SlPPD proteins display a similar domain structure. The interaction between tomato SlKIX8/SlKIX9 and the SlTPL co-repressors is expected to occur via the EAR motif

present in the SlKIX proteins (Kagale *et al.*, 2010; Causier *et al.*, 2012), as was shown for their Arabidopsis and pea orthologs (Gonzalez *et al.*, 2015; Li *et al.*, 2019).

Tomato *kix8 kix9* plants exhibited an enlarged, dome-shaped leaf phenotype, similar to the phenotype observed in Arabidopsis *kix8-kix9* and *ami-ppd* mutants (Gonzalez *et al.*, 2015). Moreover, terminal leaflets of young tomato *kix8 kix9* leaves displayed an increased expression of three putative SlPPD target genes, *SlDFL1*, *SlAHL17*, and *SlAP2d*, of which the orthologs were strongly upregulated in Arabidopsis *kix8-kix9* and *ami-ppd* leaves (Gonzalez *et al.*, 2015). Therefore, the SlPPD-SlKIX complex might restrict meristemoid division, as shown in Arabidopsis (White, 2006; Gonzalez *et al.*, 2015), during leaf development. Following the fate of meristemoids in tomato *kix8 kix9* leaves over time could help us to validate this. Taken together, we can conclude that both in rosid and asterid species, KIX8 and/or KIX9 assist PPD proteins in repressing distinct downstream target genes and in regulating leaf size and shape.

Partial redundancy of SlKIX8 and SlKIX9

In Arabidopsis, AtKIX8 and AtKIX9 were reported to have partially redundant roles in controlling leaf growth (Gonzalez *et al.*, 2015). The intermediate and absent leaf phenotype of tomato single *kix8* and *kix9* mutants, respectively, compared with the markedly enlarged, dome-shaped leaf phenotype of tomato *kix8 kix9* plants, suggests partial redundancy of SlKIX8 and SlKIX9 in tomato leaf development as well. In line with these phenotypes, transcription of *SlKIX9* is (almost) absent in most tomato tissues, whereas *SlKIX8* displays an overall higher expression level. In tomato *kix8 kix9* tomato leaflets, however, the transcript levels of not only *SlKIX8* but also *SlKIX9* were increased compared with wild-type leaflets, suggesting negative feedback of the SlPPD-SlKIX complex on the expression of both *SlKIX8* and *SlKIX9*. In yeast cells, interaction with SlTPL2 was observed for both SlKIX8 and SlKIX9, but SlKIX8 could additionally interact with SlTPL1, SlTPL4, SlTPL6, and SlSAP. A previous study showed that from the six SlTPL genes, *SlTPL1* had the highest overall expression in examined tissues and developmental stages, while *SlTPL2* was expressed to a much lesser extent (Hao *et al.*, 2014). The expression of *SlTPL4* dominated in ripening fruit and *SlTPL6* transcripts were almost absent in all investigated tissues (Hao *et al.*, 2014). Furthermore, SlTPL6 was suggested to have lost its functionality (Hao *et al.*, 2014) and, therefore, calls the biological relevance of the interaction between SlKIX8 and SlTPL6 into question. To further explore this, it could be relevant to investigate the

tissue-specific interactions between SlKIX and SlTPL proteins *in planta*. All in all, these data indicate that SlKIX8 and SlKIX9 are functionally redundant, but that SlKIX8 might play a predominant role in the regulation of leaf development.

SlKIX8 and SlKIX9 are negative regulators of fruit growth

Like any other plant organ, fruit grows by means of cell division and cell expansion. After fertilization, tomato ovary growth starts with a short period of cell proliferation followed by a longer cell expansion phase, resulting in a massive expansion of the pericarp (or fruit flesh) in particular (Xiao *et al.*, 2009). Fruit ripening commences after fruit growth is finalized. Here, we report that SlKIX8 and/or SlKIX9 act as negative regulators of fruit growth, as simultaneously knocking out *SlKIX8* and *SlKIX9* by CRISRP-Cas9 genome editing results in the production of bigger tomato fruits with increased pericarp thickness. Whether this is the consequence of prolonged cell division or cell expansion, or a combination of both still needs to be investigated.

In line with our findings, several rosid eudicot species, in which the *KIX* or *PPD* genes were either mutated or downregulated, displayed increased seed pod size (Ge *et al.*, 2016; Kanazashi *et al.*, 2018; Li *et al.*, 2019). Ge and colleagues (Ge *et al.*, 2016) attributed the larger size of pods produced by *Medicago truncatula* plants in which an *AtPPD* ortholog was mutated to a prolonged period of cell division, similar to what they observed in developing leaves. Furthermore, orthologs of AtSAP, an F-box protein that regulates the stability of the AtPPD-AtKIX complex, are positive regulators of seed pod and flower size in *Medicago truncatula*, of fruit and flower size in cucumber and of flower size in *Capsella rubella* (Sicard *et al.*, 2016; Yang *et al.*, 2018; Yin *et al.*, 2020).

Here, we show that tomato *kix8 kix9* loss-of-function lines produce enlarged fruits, which was among the main selection criteria for nearly all fruit crops during domestication and still is today (Pickersgill, 2007). Many of the alleles selected during domestication are not severe gain- or loss-of-function alleles, but are the result of mutations residing in *cis*-regulatory elements (CREs) that led to spatiotemporal expression changes of genes involved in crop development (Doebley *et al.*, 2006; Meyer & Purugganan, 2013; Swinnen *et al.*, 2016). These CRE alterations were likely favored over severe gain- or loss-of-function mutations, which would have been accompanied by undesirable pleiotropic effects (Swinnen *et al.*, 2016). The tomato *kix8 kix9* mutants displayed delayed flowering, which is an undesirable trait for

breeding that may be explained by the upregulation of *SlAP2d* in *kix8 kix9* leaves. This putative floral repressor gene is an ortholog of *AtSMZ*, which encodes a protein that counteracts the activity of CONSTANS, a promoter of flowering, by repressing the expression of multiple flowering time regulators including *FLOWERING TIME* (*FT*) in Arabidopsis leaves (Mathieu *et al.*, 2009). Engineering of CREs in the promoter regions of *SlKIX8* and *SlKIX9* to downregulate their expression specifically during fruit growth might thus present a more promising breeding strategy (Swinnen *et al.*, 2016; Rodríguez-Leal *et al.*, 2017).

Materials and Methods

Ortholog identification

Tomato protein orthologs of AtKIX8 and AtKIX9 were identified through a BLASTP search in the National Center for Biotechnology Information (NCBI) GenBank protein database. Tomato protein orthologs of AtSAP, AtDFL1, AtAHL17, and AtSMZ were retrieved from the comparative genomics resource PLAZA 4.0 Dicots (http://bioinformatics.psb.ugent.be/plaza/) (Van Bel *et al.*, 2018).

DNA Constructs

Yeast two- and three-hybrid constructs

For yeast two- (Y2H) and three-hybrid (Y3H) assays, the coding sequence of tomato *SlKIX8*, *SlKIX9*, *SlPPD1*, *SlPPD2*, and *SlSAP* was PCR-amplified with the primers listed in Table S2 and recombined in a Gateway donor vector (Invitrogen). Gateway donor vectors containing the coding sequence of tomato *SlTPL1–6* were obtained from (Hao *et al.*, 2014). Subsequently, Gateway LR reactions (Invitrogen) were performed with pGAD424gate and pGBT9gate, generating bait and prey constructs, respectively. Alternatively, MultiSite Gateway LR reactions (Invitrogen) were performed with pMG426 (Nagels Durand *et al.*, 2012) to express a third protein of interest, driven by the *GDP* promoter and C-terminally fused to the SV40 NLS-3xFLAG-6xHis tag.

CRISPR-Cas9 constructs

To select CRISPR-Cas9 guide RNA (gRNA) target sites, CRISPR-P (http://crispr.hzau.edu.cn/CRISPR/) (Lei *et al.*, 2014) was used. We selected a gRNA target site in the first exon of *SlKIX8*, whereas for *SlKIX9*, we selected a gRNA target

site in the third exon downstream of a start codon that could act as an alternative transcription start site (Fig. S2b). The CRISPR-Cas9 construct was cloned as previously described (Fauser *et al.*, 2014; Ritter *et al.*, 2017; Pauwels *et al.*, 2018). Briefly, for each gRNA target site, two complementary oligonucleotides with 4-bp overhangs (Table S2) were annealed and inserted by a Golden Gate reaction with BpiI (Thermo Scientific) and T4 DNA ligase (Thermo Scientific) in a Gateway entry vector. As Gateway entry vectors, pMR217 (L1-R5) and pMR218 (L5-L2) (Ritter *et al.*, 2017) were used. Next, a MultiSite Gateway LR reaction (Invitrogen) was used to recombine two gRNA modules with pDe-Cas9-Km (Ritter *et al.*, 2017).

Yeast two- and three-hybrid assays

Y2H and Y3H assays were performed as described previously (Cuéllar Pérez *et al.*, 2013). Briefly, for Y2H assays, the *Saccharomyces cerevisiae* PJ69-4A yeast strain was co-transformed with bait and prey constructs using the polyethylene glycol (PEG)/lithium acetate method. Transformants were selected on Synthetic Defined (SD) medium lacking Leu and Trp (−2) (Clontech). Three individual transformants were grown overnight in liquid SD (−2) medium and 10-fold dilutions of these cultures were dropped on SD control (−2) and selective medium additionally lacking His (−3) (Clontech). Empty vectors were used as negative controls. Yeast plates were allowed to grow for 2 days at 30°C before interaction was scored. Y3H assays were performed in the same way, but with different SD media compositions. For transformant selection and culturing in control media, SD medium lacking Leu, Trp, and Ura (−3) was used, whereas selective media additionally lacked His (−4) (Clontech).

Plant material and growth conditions

S. lycopersicum wild-type and CRISPR-Cas9 mutant seeds (cultivar Micro-Tom) were sown in soil. Plants were grown under long-day photoperiods (16:8). Daytime and nighttime temperatures were 26–29°C and 18–20°C, respectively.

Tomato plant transformation

Binary constructs were introduced in competent *Agrobacterium tumefaciens* (strain EHA105) cells using electroporation and transformed into *S. lycopersicum* (cultivar Micro-Tom) using the cotyledon transformation method as reported previously (Gonzalez *et al.*, 2007) with following modifications. Cotyledon pieces from one-week-

old seedlings were incubated for 24 h in de dark at 25°C on solid Murashige and Skoog (MS) medium (pH 5.7) containing 4.4 g l^{-1} of MS supplemented with vitamins (Duchefa), 20 g l^{-1} of sucrose, 0.2 g l^{-1} of KH_2PO_4, 1 mg l^{-1} of thiamine, 0.2 mM of acetosyringone, 0.2 mg l^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg l^{-1} of kinetin. Next, the cotyledon pieces were soaked in a *A. tumefaciens* (strain EHA105) bacterial suspension culture (0.05-0.10 OD) containing the binary vector for 25 min with shaking. Cotyledon pieces were dried on sterile tissue paper and placed back on the aforementioned solid MS medium for 48 h in the dark at 25°C. Cotyledon pieces were washed once with liquid MS medium (pH 5.7) containing 4.4 g l^{-1} of MS supplemented with vitamins (Duchefa), containing 20 g l^{-1} of sucrose, 0.2 g l^{-1} of KH_2PO_4, and 1 mg l^{-1} of thiamine and once with sterile water. Cotyledon pieces were dried on sterile tissue paper and placed on solid MS medium (pH 5.7) containing 4.4 g l^{-1} of MS supplemented with vitamins (Duchefa), containing 30 g l^{-1} of sucrose, 1 ml l^{-1} of 1000X Nitsch vitamin stock (for 100 ml: 0.005 g of biotin, 0.2 g of glycine, 10 g of myo-inositol, 0.5 g of nicotinic acid, 0.05 g of pyridoxine HCl, and 0.05 g of thiamine HCl), 0.5 g l^{-1} of folic acid, 2 mg l^{-1} of zeatin riboside, 100 mg l^{-1} of kanamycin, 25 mg l^{-1} of melatonin, and 300 mg l^{-1} of timentin and put in a 25°C controlled photoperiodic growth chamber (16:8 photoperiods). The medium was refreshed every 14 days until regenerated shoots appeared. These shoots were placed on solid MS medium (pH 5.7) containing 2.2 g l^{-1} of MS, 10 g l^{-1} of sucrose, 1 ml l^{-1} of 1000X Nitsch vitamin stock, 0.5 g l^{-1} of folic acid, 100 mg l^{-1} of kanamycin, and 150 mg l^{-1} of timentin until their acclimatization in the greenhouse.

Identification of CRISPR-Cas9 mutants

Plant genotyping

CRISPR-Cas9 mutants were identified as described previously (Swinnen *et al.*, 2020). Genomic DNA was prepared from homogenized leaf tissue using extraction buffer (pH 9.5) containing 0.1 M of tris(hydroxymethyl)aminomethane (Tris)-HCl, 0.25 M of KCl, and 0.01 M of ethylenediaminetetraacetic acid (EDTA). The mixture was incubated at 95°C for 10 min and cooled at 4°C for 5 min. After addition of 3% (w/v) BSA, the supernatant was used as a template in a standard PCR reaction using GoTaq (Promega) with Cas9-specific primers (to select primary plant transformant (T0) lines in which the T-DNA was present or plant T1 lines in which the T-DNA was absent) or with primers

to amplify a gRNA target region (Table S2). PCR amplicons containing a gRNA target site were purified using HighPrep PCR reagent (MAGBIO). After Sanger sequencing of the purified PCR amplicons with an amplification primer located approximately 200 bp from the Cas9 cleavage site, quantitative sequence trace data were decomposed using Tracking Indels by DEcomposition (TIDE) (https://www.deskgen.com/landing/tide.html#/tide) or Inference of CRISPR Editing (ICE) Analysis Tool (https://ice.synthego.com/#/).

Plant ploidy level analysis

Diploid CRISPR-Cas mutants (T0) were identified using flow cytometry. Leaf material (1.0 cm^2) was chopped in 200 µl of chilled CyStain UV Precise P Nuclei Extraction Buffer (Sysmex) for 2 min using a razor blade. The suspension was filtered through a 50-µm nylon filter and 800 µl of chilled CyStain UV Precise P Staining Buffer (Sysmex) was added to the isolated nuclei. The DNA content of 5,000–10,000 nuclei was measured using a CyFlow Space flow cytometer (Sysmex) and analyzed with FloMax software (Sysmex).

Phenotypic analyses

Leaf growth parameter analysis

The eighth leaf (from the top) from 2–month–old CRISPR-Cas mutant (T2) and wild-type plants was harvested for leaf growth parameter analysis. Per genotype, 31–40 biological replicates (leaves) were collected. A digital balance was used to measure the biomass/fresh weight of leaves and their terminal leaflets. Pictures of terminal leaflets were taken before (projected) and after (real) cutting the leaves to flatten them. Projected and real leaflet area were measured using ImageJ (https://imagej.nih.gov/ij/). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$).

Flowering time analysis

Flowering time of CRISPR-Cas mutant (T2) and wild-type plants was quantified by counting the number of true leaves that were produced before initiation of the primary inflorescence (Soyk *et al.*, 2017). Flowering time was measured for 15–16 plants per

genotype. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$).

Fruit growth parameter analysis

For fruit size and biomass measurements, red ripe fruits from CRISPR-Cas mutant (T2) and wild-type plants were harvested. Per genotype, 20–36 biological replicates (individual fruits) were collected. Fruit size was determined by measuring the maximum diameter of the equatorial fruit axis. To determine fruit pericarp thickness, fruits in breaker–orange (breaker + 3 days) stage were harvested and scans were taken of the equatorial sections. Per genotype, 18–20 biological replicates (individual fruits) were collected. Pericarp thickness was measured using Tomato Analyzer (version 4.0) (Brewer *et al.*, 2006). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$).

Seed parameter analysis

For seed size and number analyses, seeds were harvested from red ripe fruits produced by CRISPR-Cas mutant (T2) and wild-type plants. Per genotype, 20–36 biological replicates (fruits) were collected. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$).

Gene expression analysis by quantitative real-time PCR

The terminal leaflet of the second leaf (from the top) from 3-week-old CRISPR-Cas mutant (T2) and wild-type plants was harvested by flash freezing in liquid nitrogen and ground using the Mixer Mill 300 (Retch). Per genotype, five biological replicates, each consisting of a single terminal leaflet were collected. Messenger RNA was extracted from homogenized tissue as described in (Townsley *et al.*, 2015) with following modifications. Tissue was lysed using 800 µl of lysate binding buffer (LBB) containing 100 mM of Tris-HCl (pH 7.5), 500 mM of LiCl, 10 mM of EDTA (pH 8.0), 1% of sodium dodecyl sulfate (SDS), 5 mM of dithiothreitol (DTT), 15 µl ml^{-1} of Antifoam A, and 5 µl ml^{-1} of 2-mercaptoethanol, and the mixture was incubated for 10 min at room temperature. Messenger RNA was separated from 200 µl of lysate using 1 µl of 12.5 µM of 5' biotinylated polyT oligonucleotide (5'-biotin-ACAGGACATTCGTCGCTTCCTTTTTTTTTTTTTTTTTTT-3') and the mixture was incubated for 10 min. Next, captured messenger RNA was isolated from the lysate

by adding 20 µl of LBB-washed streptavidin-coated magnetic beads (New England Biolabs) and incubated for 10 min at room temperature. Samples were placed on a MagWell Magnetic Separator 96 (EdgeBio) and washed with 200 µl of washing buffer A (10 mM of Tris-HCl (pH 7.5), 150 mM of LiCl, 1 mM of EDTA (pH 8.0), 0.1% of SDS), washing buffer B (10 mM of Tris-HCl (pH 7.5), 150 mM of LiCl, 1 mM of EDTA (pH 8.0)), and low-salt buffer (20 mM of Tris-HCl (pH 7.5), 150 mM of NaCl, 1 mM of EDTA (pH 8.0)), all pre-chilled on ice. Elution of messenger RNA was done by adding 20 µl of 10 mM of Tris-HCl (pH 8.0) with 1 mM of 2-mercaptoethanol followed by incubation of the mixture at 80°C for 2 min.

First-strand complementary DNA was synthesized from 20 µl of messenger RNA eluate by qScript cDNA Synthesis Kit (Quantabio). Quantitative real-time PCR (qPCR) reactions were performed with a LightCycler 480 System (Roche) using Fast SYBR Green Master Mix (Applied Biosystems) and primers (Table S2) designed by QuantPrime (https://www.quantprime.de/) (Arvidsson *et al.*, 2008). Gene expression levels were quantified relative to *CLATHRIN ADAPTOR COMPLEXES MEDIUM SUBUNIT* (*SlCAC*) and *TAP42-INTERACTING PROTEIN* (*SlTIP41*) using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$).

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank/Solgenomics data libraries under the following accession numbers: *SlKIX8* (Solyc07g008100), *SlKIX9* (Solyc08g059700), *SlPPD1* (Solyc06g084120), *SlPPD2* (Solyc09g065630), *SlSAP* (Solyc05g041220), *SlDFL1* (Solyc07g063850), *SlAHL17* (Solyc04g076220), *SlAP2d* (Solyc11g072600), *SlCAC* (Solyc08g006960), and *SlTIP41* (Solyc10g049850).

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Author Contribution

G.S., A.B., L.P., and A.G. designed the experiments. G.S., A.B., R.D.C., and J.V.D. performed the experiments. G.S., A.B., L.P., and A.G. analyzed the data. G.S. wrote the article and A.B., L.P., A.G. and N.G. revised the manuscript and contributed to the writing. L.P., A.G., and D.I. supervised the project.

Supporting Information

Additional supporting information may be found in the Supporting Information section at the end of this article.

Fig. S1 A conserved repressor complex regulates leaf growth in distinct eudicot species.
Fig. S2 Splice variants of *SlKIX8*, *SlKIX9*, *SlPPD1*, and *SlPPD2*.
Fig. S3 Regenerated tomato *kix8 kix9* plants display a rippled, dome-shaped leaf phenotype.
Fig. S4 CRISPR-Cas9 mutations in double *kix8 kix9* (T1), single *kix8*, and single *kix9* tomato knockout lines.
Fig. S5 Tomato *kix8 kix9* plants do not exhibit a seed phenotype.
Table S1 Normalized expression of *SlKIX8*, *SlKIX9*, *SlPPD1*, *SlPPD2*, *SlDFL1*, *SlAHL17,* and *SlAP2d* in different tomato organs and developmental stages (cultivar Micro-Tom) used to draw heat maps in Fig. 4a–b.
Table S2 Oligonucleotides used in this study.

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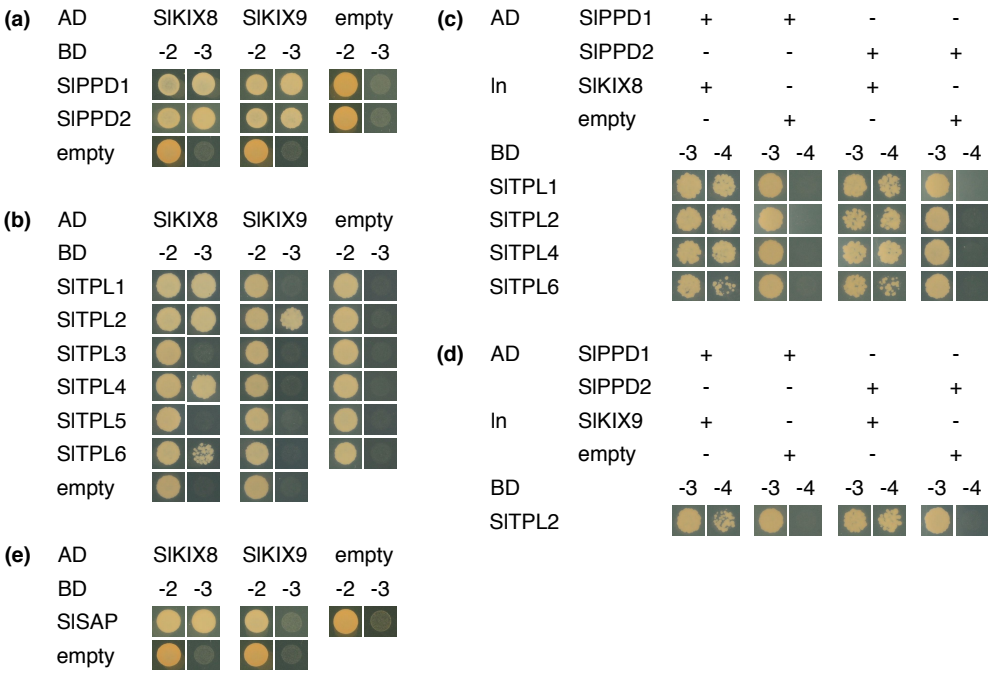

Fig. 1 SlKIX8 and SlKIX9 are SlTPL adaptors for SlPPD proteins. (a−b) Y2H interaction analysis of SlKIX8 and SlKIX9 with SlPPD (a) and SlTPL (b) proteins. Yeast transformants expressing bait (BD) and prey (AD) proteins were dropped on control medium lacking Leu and Trp (−2) or selective medium additionally lacking His (−3). (c-d) Y3H interaction analysis to test the bridging capacity of SlKIX8 (c) and SlKIX9 (d) to mediate the SlPPD-SlTPL interaction. Yeast transformants expressing bait (BD), bridge (ln), and prey (AD) proteins were dropped on control medium lacking Leu, Trp, and Ura (−3) or selective medium additionally lacking His (−4). (e) Y2H interaction analysis of SlKIX proteins with SlSAP. Yeast transformants expressing bait (BD) and prey (AD) proteins were dropped on control medium lacking Leu and Trp (−2) or selective medium additionally lacking His (−3). Empty vectors were used for all control assays. Abbreviations: AD, activation domain; BD, binding domain; ln, linker.

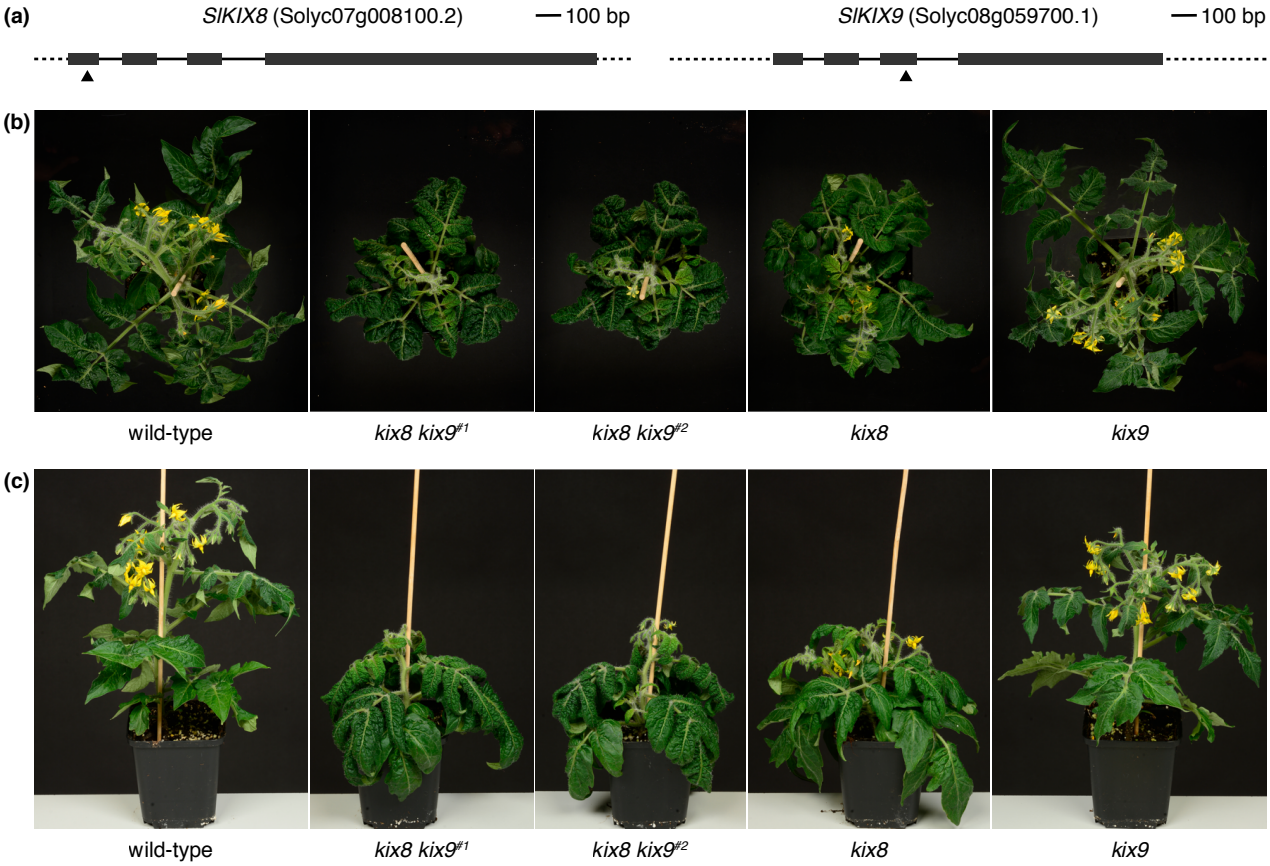

Fig. 2 CRISPR-Cas9 genome editing of tomato *SlKIX8* and *SlKIX9* causes a rippled, dome-shaped leaf phenotype. (a) Schematic representation of *SlKIX8* and *SlKIX9* with location of the CRISPR-Cas9 cleavage sites. Dark grey boxes represent exons. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. (b−c) Representative wild-type, *kix8 kix9*$^{\#1}$, *kix8 kix9*$^{\#2}$, *kix8*, and *kix9* plants grown in soil for 1 month under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively, were photographed from the top (b) and the front (c).

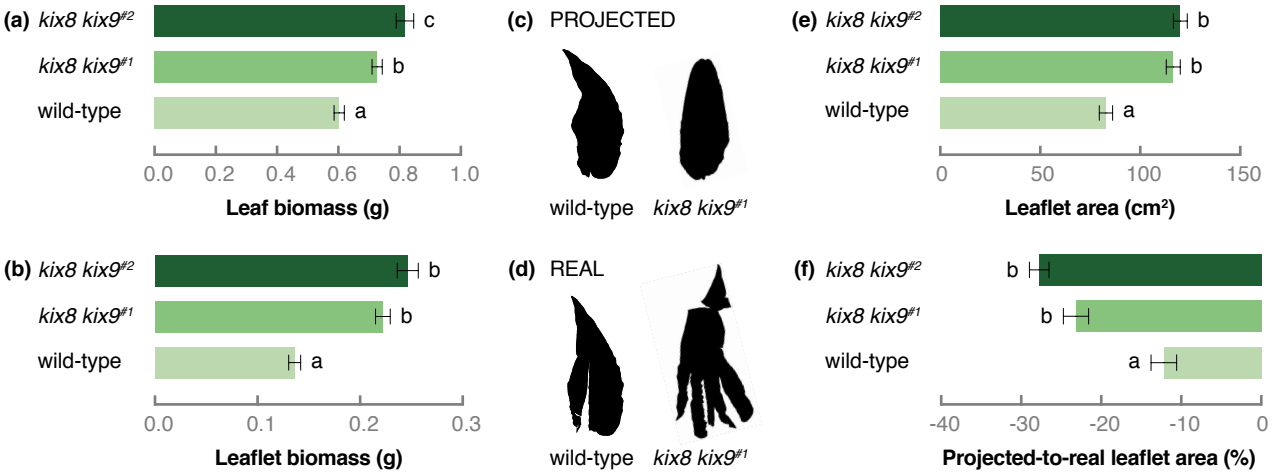

Fig. 3 Tomato *kix8 kix9* plants produce enlarged, dome-shaped leaves. (a−b) Biomass of leaf eight (from the top) (a) and its terminal leaflet (b). The eighth leaf (from the top) was harvested from plants grown in soil for 2 months under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively. Bars represent mean biomass relative to the mean of wild-type biomass values. Error bars denote standard error (n = 31–40). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters). (c−d) The terminal leaflet area was measured before (projected, c) and after (real, d) the terminal leaflet of the eighth leaf was cut to flatten it. (e−f) Area (e) and projected-to-real area (f) of the terminal leaflet of the eighth leaf. Bars represent mean area relative to the mean of wild-type area values. Error bars denote standard error (n = 31–40). Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters).

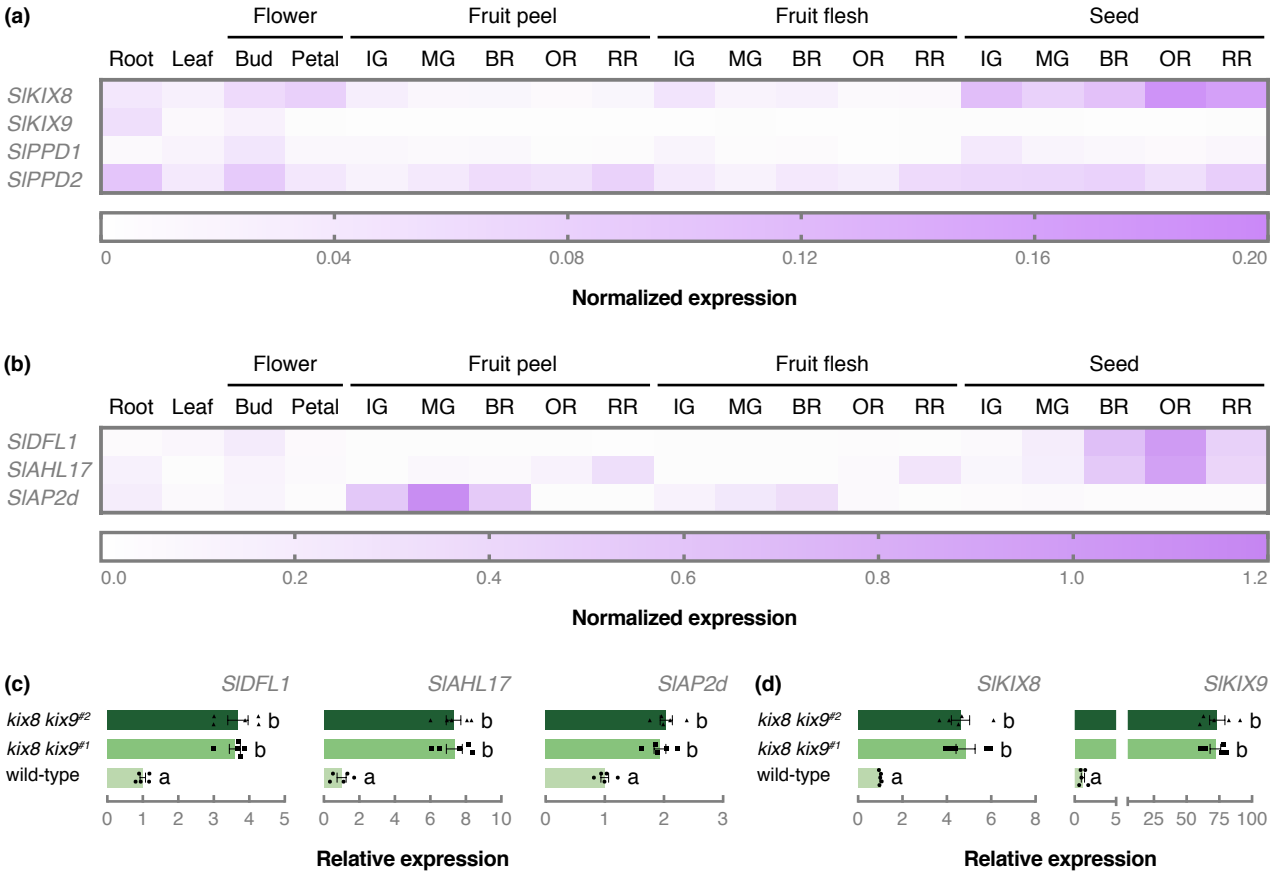

Fig. 4 SlKIX8 and SlKIX9 are required for the repression of putative SlPPD target genes in tomato. (a–b) Normalized expression profiles of *SlKIX8*, *SlKIX9*, *SlPPD1*, *SlPPD2* (a), *SlDFL1*, *SlAHL17*, and *SlAP2d* (b) in different tomato organs and developmental stages (cultivar Micro-Tom). Expression data was obtained from TomExpress (Zouine *et al.*, 2017) and can be found in Table S1. (c–d) Relative expression of *SlDFL1*, *SlAHL17*, *SlAP2d* (c), *SlKIX8*, and *SlKIX9* (d) in terminal leaflets of not fully developed leaves analyzed by qPCR. The terminal leaflet from the second leaf (from the top) was harvested from plants grown in soil for 3 weeks under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively. Bars represent mean expression relative to the mean of wild-type expression values. Error bars denote standard error (n = 5). Individual wild-type (●), *kix8 kix9*$^{\#1}$ (■), and *kix8 kix9*$^{\#2}$ (▲) values are shown. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters). Abbreviations: IG, immature green; MG, mature green; BR, breaker; OR, orange; RR, red ripe.

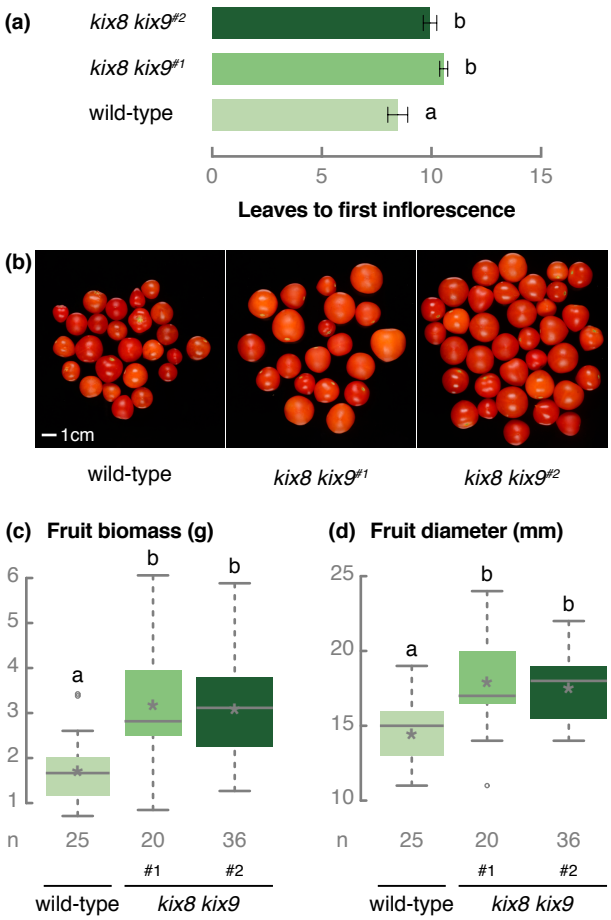

Fig. 5 Tomato *kix8 kix9* plants produce bigger fruits. (a) Flowering time measured by the number of leaves produced before initiation of the first inflorescence. (b) Red ripe fruits produced by wild-type, *kix8 kix9*$^{\#1}$, and *kix8 kix9*$^{\#2}$ plants. (c−d) Biomass (c) and diameter (d) of red ripe fruits. Boxes span the interquartile range with whiskers extending 1.5 times the interquartile range. The median and mean are indicated by the center line and asterisks, respectively. Outliers are represented by open circles. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters). For all analyses, plants were grown in soil under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively.

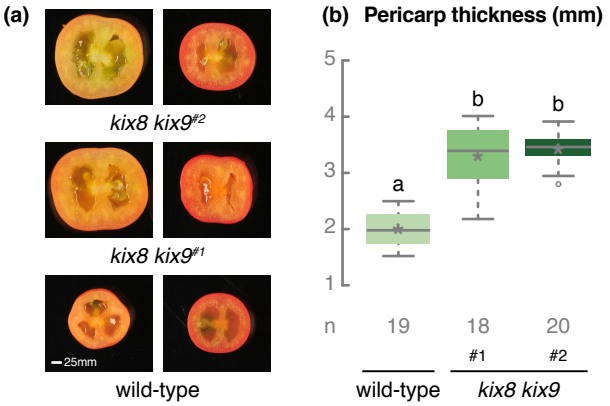

Fig. 6 Fruits produced by *kix8 kix9* plants display increased pericarp thickness. (a) Equatorial sections of representative orange and red ripe fruits produced by wild-type, *kix8 kix9*$^{\#1}$, and *kix8 kix9*$^{\#2}$ plants. (b) Pericarp thickness of breaker–orange fruits. Plants were grown in soil under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively. Boxes span the interquartile range with whiskers extending 1.5 times the interquartile range. The median and mean are indicated by the center line and asterisks, respectively. Outliers are represented by open circles. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters).

Supporting Information

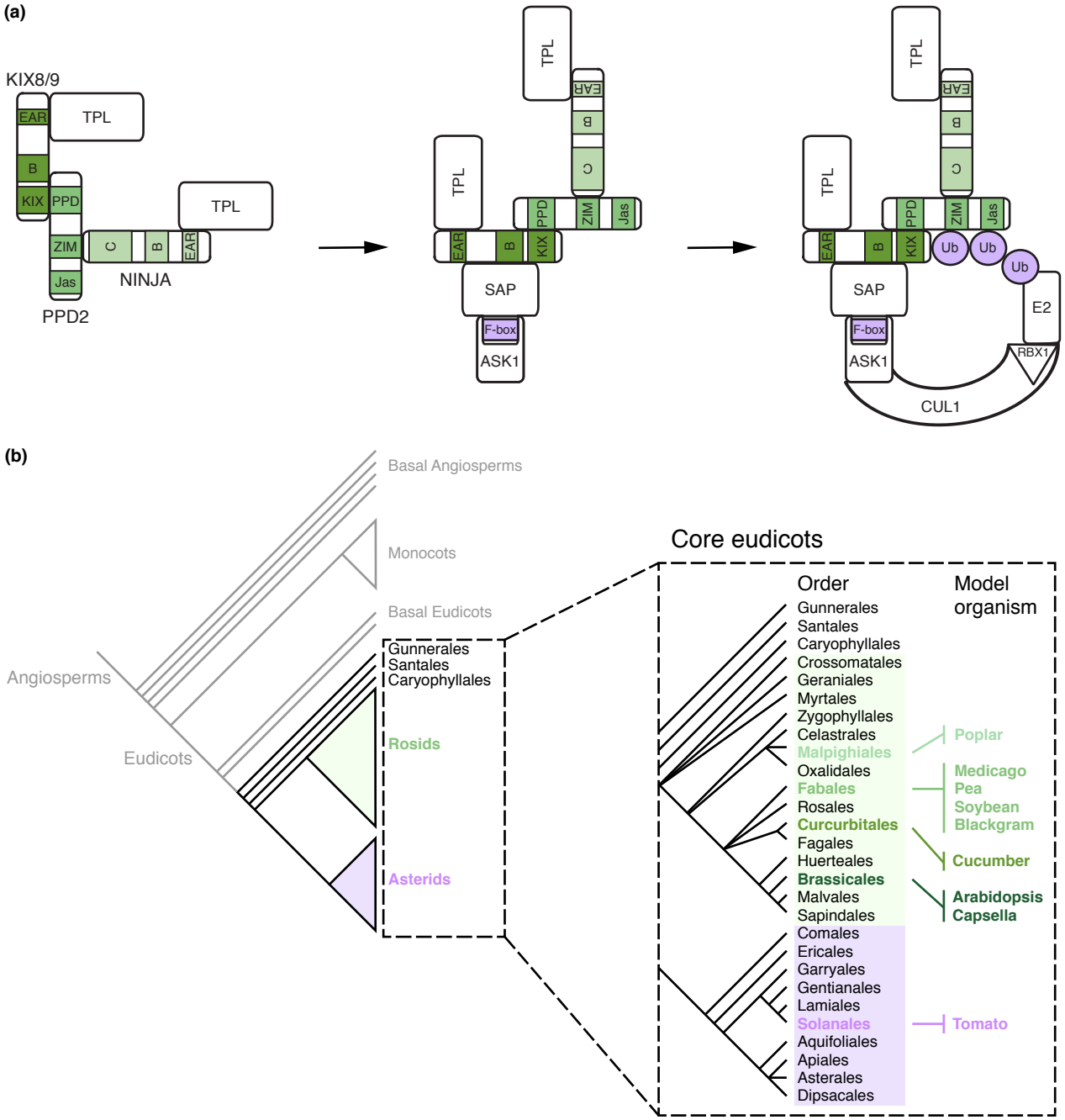

Fig. S1 A conserved repressor complex regulates leaf growth in distinct eudicot species. (a) The AtPPD2-AtKIX8/AtKIX9 transcriptional repressor complex in *Arabidopsis thaliana*. AtPPD2 interacts with AtKIX8/AtKIX9 and AtNINJA to recruit AtTPL. Interaction of repressor complex members with the E3 ubiquitin ligase AtSCF^{SAP} (comprising the F-box protein AtSAP, AtASK1, AtCUL1, and AtRBX1) leads to the proteasomal degradation of AtKIX8/AtKIX9 and AtPPD2. (b) Model organisms in which KIX, PPD and/or SAP proteins were shown to mediate leaf growth belong to different orders within the rosids, which together with the asterids, make up most of the core eudicot species. Tomato is an asterid model species in which the potential role of these proteins in regulating leaf growth has not been investigated yet. Abbreviations: ASK1, Arabidopsis SKP1; CUL1, CULLIN 1; RBX1, RING-BOX 1; SKP1, S-PHASE KINASE-ASSOCIATED PROTEIN 1; Ub, ubiquitin.

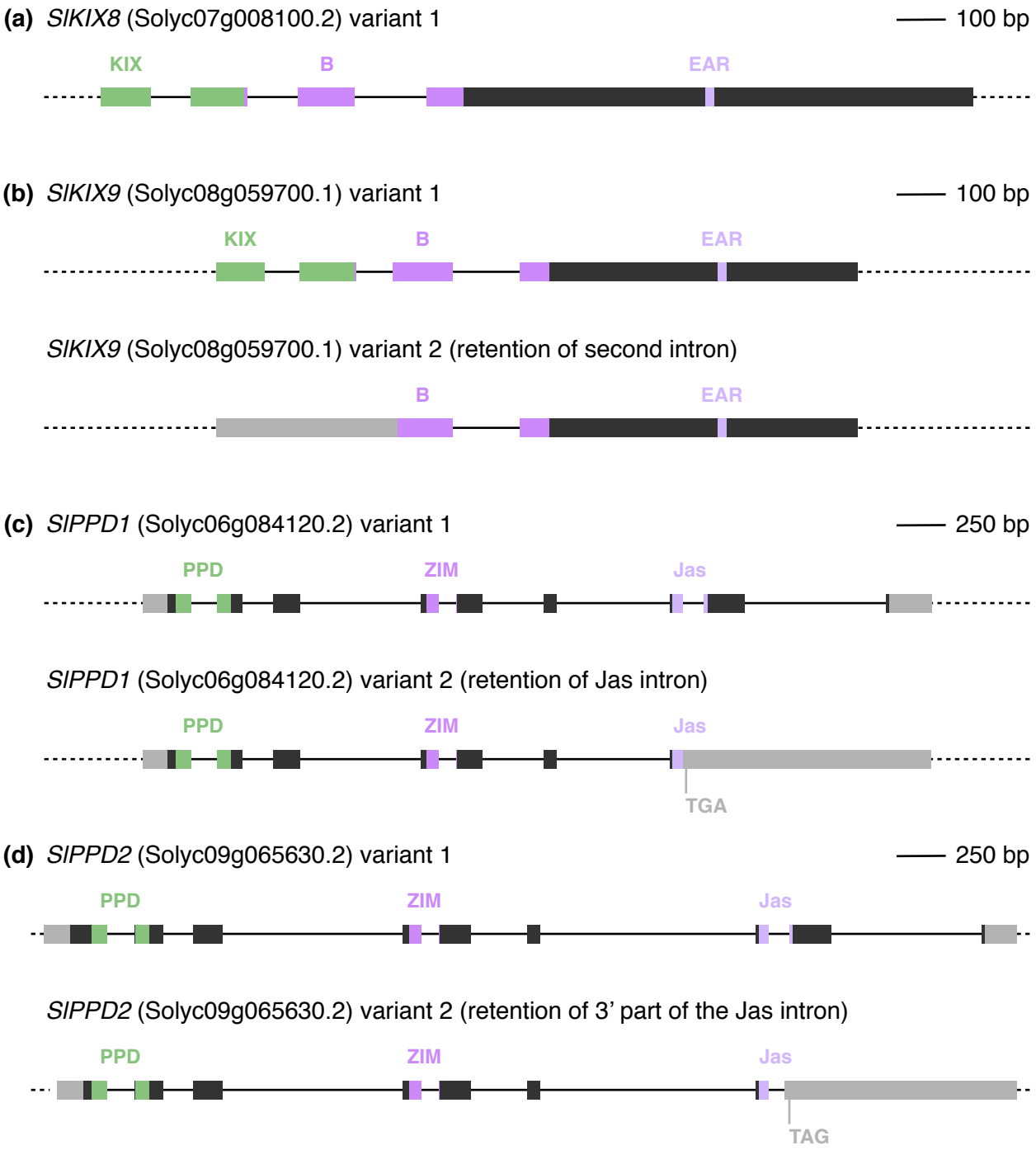

Fig. S2 Splice variants of *SlKIX8*, *SlKIX9*, *SlPPD1*, and *SlPPD2*. Dark grey boxes represent exons, solid lines represent introns and light grey boxes represent UTRs. Green and purple boxes represent encoded protein domains. No alternative splicing was observed for *SlKIX8* (a). Retention of the second *SlKIX9* intron (b) could lead to the use of a downstream start codon, excluding the sequence that encodes the N-terminal KIX domain. The splice variants of *SlPPD1* (c) and *SlPPD2* (d) display retention of the Jas intron and part of the Jas intron, respectively, which is located between the two exons that encode the Jas domain. These alternative splicing events generate premature stop codons. Abbreviations: UTR, untranslated region.

Fig. S3 Regenerated tomato *kix8 kix9* plants display a rippled, dome-shaped leaf phenotype. (a–b) Wild-type and regenerated *kix8 kix9* plants were photographed from the front (a) and the top (b). Primary transformants transferred from rooting medium were grown in soil for 10 weeks under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively.

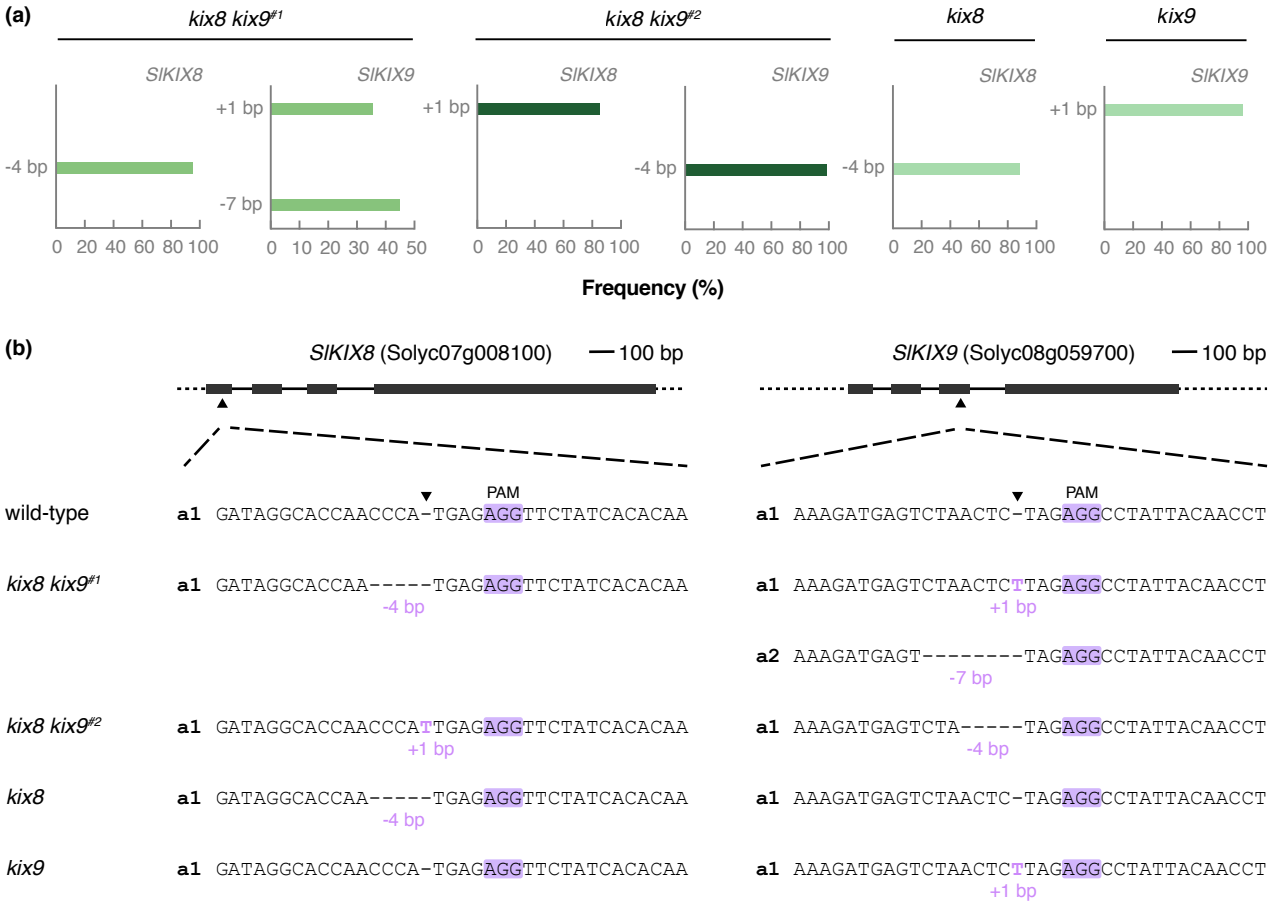

Fig. S4 CRISPR-Cas9 mutations in double *kix8 kix9* (T1) and single *kix8*, and single *kix9* tomato knockout lines. (a) ICE analysis of genomic sites targeted by the guide RNAs. Targeted genomic regions were PCR amplified and sequenced by Sanger sequencing. Based on the sequence chromatograms, ICE analysis visualized the indel spectrum and calculated the frequency of each indel. (b) Schematic representation of *SlKIX8* and *SlKIX9* with location of the CRISPR-Cas9 cleavage sites. Dark grey boxes represent exons and solid lines represent introns. Cas9 cleavage sites for guide RNAs are indicated with arrowheads. Allele sequences are shown for two independent *kix8 kix9* lines and one *kix8* and one *kix9* line. Abbreviations: a, allele; PAM, protospacer adjacent motif.

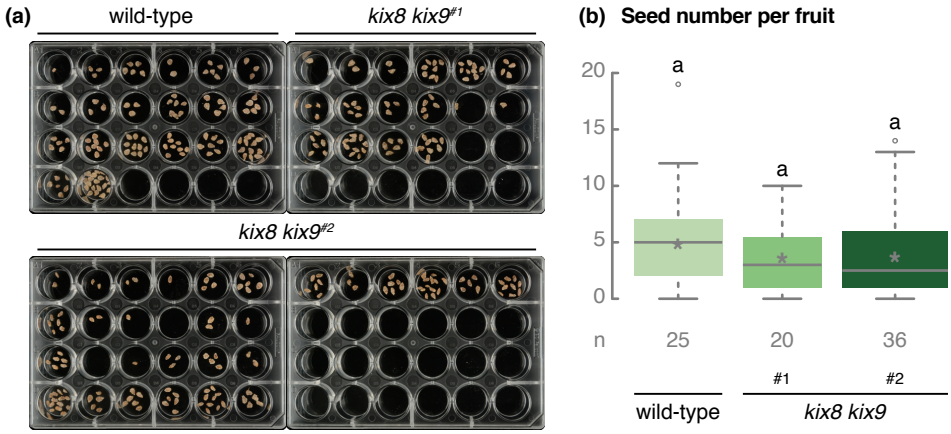

Fig. S5 Tomato *kix8 kix9* plants do not exhibit a seed phenotype. (a) Seeds produced by wild-type, *kix8 kix9*$^{\#1}$, and *kix8 kix9*$^{\#2}$ plants. Each well contains the seeds produced by a single fruit. (b) Number of seeds produced by wild-type, *kix8 kix9*$^{\#1}$, and *kix8 kix9*$^{\#2}$ plants. Boxes span the interquartile range with whiskers extending 1.5 times the interquartile range. The median and mean are indicated by the center line and asterisks, respectively. Outliers are represented by open circles. Statistical significance was determined by ANOVA followed by Tukey post-hoc analysis ($P < 0.05$; indicated by different letters). Plants were grown in soil under 16:8 photoperiods with daytime and nighttime temperatures of 26–29°C and 18–20°C, respectively.

Table S1 Normalized expression of *SlKIX8*, *SlKIX9*, *SlPPD1*, *SlPPD2*, *SlDFL1*, *SlAHL17*, and *SlAP2d* in different tomato organs and developmental stages (cultivar Micro-Tom) used to draw heat maps in Fig. 4a–b.

			Flower		Fruit peel					Fruit flesh					Seed				
	Root	Leaf	Bud	Petal	IG	MG	BR	OR	RR	IG	MG	BR	OR	RR	IG	MG	BR	OR	RR
SlKIX8	0.040	0.022	0.061	0.079	0.026	0.011	0.013	0.006	0.0134	0.044	0.018	0.022	0.008	0.009	0.120	0.078	0.103	0.179	0.158
SlKIX9	0.053	0.008	0.021	0	0	0	0	0	0	0	0.001	0	0	0	0.001	0.001	0	0	0
SlPPD1	0.006	0.017	0.041	0.010	0.010	0.006	0.010	0	0	0.020	0.003	0.005	0	0.002	0.035	0.015	0.011	0.006	0.012
SlPPD2	0.098	0.035	0.090	0.039	0.020	0.034	0.060	0.050	0.080	0.040	0.020	0.037	0.029	0.061	0.066	0.069	0.074	0.053	0.082
SlDFL1	0.031	0.067	0.190	0.033	0.007	0.011	0	0	0	0.010	0.007	0.007	0.019	0.004	0.049	0.165	0.633	0.980	0.450
SlAHL17	0.123	0	0.098	0.043	0.010	0.065	0.040	0.110	0.310	0.010	0.017	0.015	0.041	0.253	0.071	0.158	0.533	0.925	0.405
SlAP2d	0.164	0.046	0.092	0.024	0.542	1.108	0.520	0	0	0.120	0.223	0.323	0.036	0.003	0.032	0.038	0.019	0.023	0.025

Expression data was obtained from TomExpress (Zouine *et al.*, 2017). Abbreviations: IG, immature green; MG, mature green; BR, breaker; OR, orange; RR, red ripe.

Table S2 Oligonucleotides used in this study.

Oligonucleotide	Sequence (5'–3')	Orientation	Description	SolycID
Oligonucleotides for Y2H/Y3H constructs:				
LAPAU2860	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCCTAGACCAGGACCCAG	Forward	amplification of *SlKIX8*	Solyc07g008100.2
LAPAU2994	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCMCAAACCTGGCCTTTTCATTTG	Reverse		
LAPAU2862	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCCTAAATCTACAAGAGC	Forward	amplification of *SlKIX9*	Solyc08g059700.1
LAPAU2863	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCMGGACTTGAATTTGTTAAAATG	Reverse		
LAPAU2856	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCCGCCGGAAGAAACAG	Forward	amplification of *SlPPD1*	Solyc06g084120.2
LAPAU2857	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCMCTTTCTAACATCTCTGTC	Reverse		
LAPAU2858	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCGCTGGAACAAACTG	Forward	amplification of *SlPPD2*	Solyc09g065630.2
LAPAU2859	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCMCTCTTTACCATCTTTG	Reverse		
COMBI6198	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCGTCTTCACAATCACCACCATC	Forward	amplification of *SlSAP*	Solyc05g041220.2
COMBI6199	GGGGACCACTTTGTACAAGAAAGCTGGGTTCACTATTGTGCACCAAAGTCCCACAAATG	Reverse		
Oligonucleotides for CRISPR-Cas9 constructs:				
LAPAU2582	ATTGATAGGCACCAACCCATGAG	Forward	*SlKIX8* gRNA target site	Solyc07g008100.2
LAPAU2583	AAACCTCATGGGTTGGTGCCTAT	Reverse		
LAPAU2580	ATTGAAAGATGAGTCTAACTCTAG	Forward	*SlKIX9* gRNA target site	Solyc08g059700.1
LAPAU2581	AAACCTAGAGTTAGACTCATCTTT	Reverse		
Oligonucleotides for the identification of CRISPR-Cas9 mutants:				
LAPAU3075	TCCCTCATCAGATCCACCTC	Forward	amplification of *Cas9*	–
LAPAU3076	CTGAAACCTGAGCCTTCTGG	Reverse		
LAPAU2783	CCCCTCCAAAACACTCATGT	Forward	amplification of *SlKIX8* gRNA target region	Solyc07g008100.2
LAPAU2784	GAGCAGTACAAATGAGCAGCA	Reverse		
LAPAU2785	GCTGAAGAAATTATGTATTCCAAAGC	Forward	amplification of *SlKIX9* gRNA target region	Solyc08g059700.1
LAPAU2786	CCCGAGAAGTTTCACTCGAA	Reverse		
Oligonucleotides for gene expression analysis by qPCR:				
COMBI5428	CCTCCGTTGTGATGTAACTGG	Forward	amplification of *SlCAC*	Solyc08g006960.2
COMBI5429	ATTGGTGGAAAGTAACATCATCG	Reverse		
COMBI5416	ATGGAGTTTTTGAGTCTTCTGC	Forward	amplification of *SlTIP41*	Solyc10g049850.1
COMBI5417	GCTGCGTTTCTGGCTTAGG	Reverse		
COMBI7162	ACCATCGAAGAGTCTCTCAACAGC	Forward	amplification of *SlDFL1*	Solyc07g063850.2
COMBI7163	CAATGGATTTGTCTGAGGCACGAC	Reverse		
COMBI7168	CTGTCATTTGCCGTCGGATGTG	Forward	amplification of *SlAHL17*	Solyc04g076220.2
COMBI7169	AGTAAGGCGGTGGTTGTGGTTG	Reverse		

COMBI7158	TGCATAGTCAGGTCGGAACAACG	Forward	amplification of *SlAP2d*	Solyc11g072600.1
COMBI7159	TGGTAGCCGGAGTTGAGAATCC	Reverse		
COMBI7188	AGGCTGTGTCTACCAGCAAAGAC	Forward	amplification of *SlKIX8*	Solyc07g008100.2
COMBI7189	TTGCAACCCGGAGTGACTGTTG	Reverse		
COMBI7190	AGACACCAACCAATCAGAGGTTCC	Forward	amplification of *SlKIX9*	Solyc08g059700.1
COMBI7191	TGCTGAGCCATGAACCTCATTCAC	Reverse		