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Overlapping functions of YDA and MAPKKK3/MAPKKK5 upstream of MPK3/MPK6 in plant immunity and growth/development[oo]

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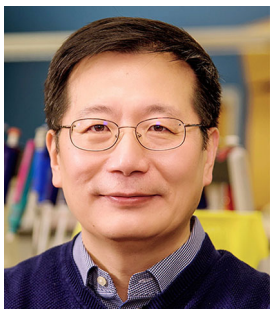

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ABSTRACT

Arabidopsis MITOGEN-ACTIVATED PROTEIN KINASE3 (MAPK3 or MPK3) and MPK6 play important signaling roles in plant immunity and growth/development. MAPK KINASE4 (MKK4) and MKK5 function redundantly upstream of MPK3 and MPK6 in these processes. YODA (YDA), also known as MAPK KINASE KINASE4 (MAPKKK4), is upstream of MKK4/MKK5 and forms a complete MAPK cascade (YDA–MKK4/MKK5–MPK3/MPK6) in regulating plant growth and development. In plant immunity, MAPKKK3 and MAPKKK5 function redundantly upstream of the same MKK4/MKK5–MPK3/MPK6 module. However, the residual activation of MPK3/MPK6 in the *mapkkk3 mapkkk5* double mutant in response to flg22 pathogen-associated molecular pattern (PAMP) treatment suggests the presence of additional MAPKKK(s) in this MAPK cascade in signaling plant immunity. To investigate whether YDA is also involved in plant immunity, we attempted to generate *mapkkk3 mapkkk5 yda* triple mutants. However, it was not possible to recover one of the double mutant combinations (*mapkkk5 yda*) or the triple mutant (*mapkkk3 mapkkk5 yda*) due to a failure of embryogenesis. Using the clustered regularly interspaced short palindromic repeats (CRISPR) – CRISPR-associated protein 9 (Cas9) approach, we generated weak, N-terminal deletion alleles of *YDA*, *yda-del*, in a *mapkkk3 mapkkk5* background. PAMP-triggered MPK3/MPK6 activation was further reduced in the *mapkkk3 mapkkk5 yda-del* mutant, and the triple mutant was more susceptible to pathogen infection, suggesting YDA also plays an important role in plant immune signaling. In addition, *MAPKKK5* and, to a lesser extent, MAPKKK3 were found to contribute to gamete function and embryogenesis, together with *YDA*. While the double homozygous *mapkkk3 yda* mutant showed the same growth and development defects as the *yda* single mutant, *mapkkk5 yda* double mutant and *mapkkk3 mapkkk5 yda* triple mutants were embryo lethal, similar to the *mpk3 mpk6* double mutants. These results demonstrate that YDA, MAPKKK3, and MAPKKK5 have overlapping functions upstream of the MKK4/MKK5–MPK3/MPK6 module in both plant immunity and growth/development.

Keywords: embryogenesis, gamete transmission, MAPK cascade, MPK3/MPK6, MAPKKK3/MAPKKK5, plant immunity, YDA

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INTRODUCTION

Mitogen-activated protein kinase (MAPK) cascades are important signaling modules in all eukaryotes (Widmann et al., 1999; Ichimura et al., 2002; Zhang and Zhang, 2022). A typical MAPK cascade has at least one MAPK (or MPK), one MAPK kinase (MAPKK, also known as MKK or MEK), and one MAPKK kinase (MAPKKK, also known as MKKK or MEKK). Multiple members playing redundant or partially overlapping functions may be present at the same tier of the cascade. In response to a stimulus, the activation of MAPKKK(s), the topmost kinase(s) in a MAPK cascade, results in the phosphorylation activation of the downstream MAPKK(s). The activated MAPKK(s) then phosphorylate and activate the MAPK(s), which are capable of phosphorylating multiple downstream substrates, including transcription factors, protein kinases, other enzymes, and structural proteins, leading to a change in cellular physiology (reviewed in Ichimura et al., 2002; Pedley and Martin, 2005; Colcombet and Hirt, 2008; Meng and Zhang, 2013; Xu and Zhang, 2015; Bi and Zhou, 2017; Zhang et al., 2018; Sun and Zhang, 2022).

An increasing body of evidence has demonstrated that plant MAPK cascades are key signaling modules downstream of receptors/sensors. In plant growth and development, they function downstream of many receptor-like protein kinases to coordinate cellular responses to achieve normal growth and development in response to internally produced peptide ligands (reviewed in Xu and Zhang, 2015; Zhang et al., 2018; Sun and Zhang, 2022; Zhang and Zhang, 2022). Plant MAPK cascades are also key to the plant response to pathogen invasion by translating the signals generated from plant cell-surface pattern-recognition receptors (PRRs) and intracellular immune receptors with nucleotide-binding and leucine-rich domains after sensing pathogen-derived pathogen-associated molecular patterns (PAMPs) and pathogen-derived effectors, respectively. In addition, plant MAPK cascades are also involved in transmitting plant-derived damage-associated molecular patterns to send an early warning to other parts of the plant (Bi and Zhou, 2017; Sun and Zhang, 2022; Zhang and Zhang, 2022).

Among the 20 MAPKs in *Arabidopsis*, MPK3 and MPK6 have received the most attention because of the ease of detecting their rapid activation in response to a diverse array of abiotic and abiotic stress-related stimuli (reviewed in Zhang and Klessig, 2001; Sun and Zhang, 2022; Zhang and Zhang, 2022). In the process of acquiring a loss-of-function system for the functional analysis of *MPK3* and *MPK6* in plant immunity, we discovered that the loss of both *MPK3* and *MPK6* genes leads to embryonic lethality. In addition, they play redundant/overlapping functions in a number of other growth and developmental processes, including stomatal development, abscission, gametogenesis, pollen guidance, inflorescence architecture, seed formation, and root development (Wang et al., 2007; Cho et al., 2008; Meng et al., 2012; Guan et al., 2014a, 2014b; Zhang et al., 2017; Zhu et al., 2019; Lu et al., 2020; Shao et al., 2020). Two *Arabidopsis* MAPKKs, MKK4 and MKK5, are upstream of MPK3/MPK6 in all these processes. YODA (YDA), also known as MAPKKK4, has been shown to be the MAPKKK in the YDA–MKK4/MKK5–MPK3/MPK6 MAPK cascade in signaling plant growth and development (reviewed in Sun and Zhang, 2022; Zhang and Zhang, 2022).

In plant immunity, MAPKKK3 and MAPKKK5 have been reported to be the upstream MAPKKKs of the MKK4/MKK5-MPK3/MPK6 module, forming a complete MAPK cascade composed of MAPKKK3/MAPKKK5–MKK4/MKK5–MPK3/MPK6 (Bi et al., 2018; Sun et al., 2018). *Arabidopsis* MAPKKK3 is an ortholog of tobacco MAPKKKα which has been shown to be upstream of NtMEK2 and salicylic acid-induced protein kinase (SIPK), tobacco orthologs of MKK4/MKK5 and MPK6, respectively, in the plant hypersensitive response and pathogen resistance (del Pozo et al., 2004). These lead to the speculation that different MAPKKKs, such as YDA and MAPKKK3/MAPKKK5, might be upstream of the same MKK4/MKK5–MPK3/MPK6 module to form two separate MAPK cascades in signaling plant growth/development and immunity, respectively. However, only a partial loss of MPK3/MPK6 activation was observed in the *mapkkk3 mapkkk5* double mutant in response to PAMPs (Bi et al., 2018; Sun et al., 2018), suggesting the existence of additional MAPKKK(s) that might be functionally redundant with MAPKKK3 and MAPKKK5 in plant immunity (Bi et al., 2018; Sun et al., 2018). In addition, the loss-of-function *yda* mutant shows weaker developmental phenotypes in comparison to the *mpk3 mpk6* double mutant. For instance, *yda* homozygous seedlings can be recovered in the progenies of *yda*/+ plants (Lukowitz et al., 2004; Wang et al., 2007). In contrast, *mpk3 mpk6* double mutant cannot be recovered from the progenies of either *mpk3 mpk6*/+ or *mpk3*/+ *mpk6* plants, and *mpk3 mpk6* double mutant embryos abort very early in development (Wang et al., 2007). Together, these observations reinforce the possibility that there are additional MAPKKK(s) besides YDA in the MPK3/MPK6 MAPK cascade in signaling plant growth and development as well.

In this report, we demonstrate that *MAPKKK3*/*MAPKKK5* and *YDA* play overlapping functions in both plant immunity and growth/development. Phylogenetic analysis shows that YDA is closely related to MAPKKK3 and MAPKKK5 (Figure S1). They form a single unique clade in the *Arabidopsis* MEKK-subfamily of MAPKKKs. We attempted to generate *mapkkk3 mapkkk5 yda* triple mutants using two independent approaches: (1) crossing of the *mapkkk3 mapkkk5* double mutant with the heterozygous *yda*/+ knockout mutant; and (2) clustered regularly interspaced short palindromic repeats (CRISPR) – CRISPR-associated protein 9 (Cas9) knockout of *YDA* in the *mapkkk3 mapkkk5* double mutant background. When the *yda* knockout mutant allele (SALK_105078) was used for crossing, no *mapkkk5 yda* double or *mapkkk3 mapkkk5 yda* triple mutants were identified in the F2 and F3 generations. In contrast, *mapkkk3 yda* double mutant progenies were identified and had the same growth and developmental defects as the *yda* single mutant, suggesting *MAPKKK3* plays a minimal role in the process. When the CRISPR-Cas9 approach was used, we recovered only *yda* weak mutant alleles with in-frame deletions (*yda-del* mutants),

suggesting that frame-shifting knockout mutants of *yda* in the *mapkkk3 mapkkk5* background might be lethal, consistent with the results from crossing the *yda* knockout mutant and *mapkkk3 mapkkk5* double mutant. In addition to the growth/developmental phenotypes, an *yda-del* allele (*yda-Δ42* with 42 amino acids deleted) in the *mapkkk3 mapkkk5* background further compromised the activation of MPK3 and MPK6 in response to PAMP treatment and plant resistance against *Pseudomonas syringae pv. tomato DC3000* (*Pst*). Based on these findings, we conclude that *YDA*, *MAPPKKK3*, and *MAPKKK5* play overlapping functions in both plant immunity and growth/development. Their differential contribution to a specific process is hypothesized to be dependent on their levels of expression in particular cells/tissues/organs.

RESULTS

No homozygous double or triple mutant plants can be recovered from the progenies of *mapkkk5 yda*/+ or *mapkkk3 mapkkk5 yda*/+ plants

YDA functions upstream of MKK4/MKK5–MPK3/MPK6 to form a complete MAPK cascade in regulating a variety of plant growth and developmental processes, including embryogenesis, stomatal differentiation, and root development (reviewed in Xu and Zhang, 2015; Sun and Zhang, 2022; Zhang and Zhang, 2022). Homozygous *yda* knockout (SALK_105078) seedlings are severely dwarfed and cannot survive in soil or set seeds (Lukowitz et al., 2004; Wang et al., 2007). In the *yda* homozygous seedlings, the activation of MPK3/MPK6 in response to flg22 treatment was not compromised (Figure S2), a likely result of the presence of *MAPKKK3* and *MAPKKK5,* which have been identified as two key MAPKKKs in the MPK3/MPK6 cascade downstream of PRRs in plant immunity (Bi et al., 2018; Sun et al., 2018). However, the residual activation of MPK3/MPK6 in the *mapkkk3 mapkkk5* double mutant also indicates the existence of additional MAPKKK(s) in the MPK3/MPK6 MAPK cascade in plant immune signaling. To determine whether YDA is also involved in the activation of MPK3/MPK6 in plant immunity, we attempted to generate a *mapkkk3 mapkkk5 yda* triple mutant by crossing the *mapkkk3 mapkkk5* double mutant with *yda*/+ heterozygous plants. We envisioned that the triple mutant might be similar to the *yda* single mutant in growth and development since *mapkkk3 mapkkk5* double mutant plants have a wild-type appearance. This would allow us to recover triple homozygous mutant seedlings from the *mapkkk3 mapkkk5 yda*/+ segregating population for testing the activation of MPK3/MPK6 in response to flg22 PAMP treatment.

However, no triple homozygous *mapkkk3 mapkkk5 yda* progeny could be identified in the segregating F2 population. We then screened progenies from *mapkkk3 yda*/+, *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ plants. Double homozygous *mapkkk3 yda* seedlings were identified among the progenies of *mapkkk3 yda*/+ plants and were indistinguishable from *yda* in morphology (Figure 1A). In addition to a severely dwarfed stature, both *yda* and *mapkkk3 yda* seedlings had a severe stomatal clustering phenotype (Figure 1B–D). In contrast, no *mapkkk5 yda* or *mapkkk3 mapkkk5 yda* seedlings could be recovered from *mapkkk5 yda*/+ or *mapkkk3 mapkkk5 yda*/+ plants, suggesting potential defect(s) in either gamete transmission or embryogenesis or both.

We then characterized the segregation patterns by genotyping the progenies from *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ plants. As shown in Table 1, double homozygous *mapkkk3 yda* progenies were recovered from the progenies of *mapkkk3 yda*/+ plants at a similar frequency as *yda* homozygous seedlings from *yda*/+ plants (both were less than 25%). The reduced frequency of *yda* homozygotes in either the wild-type or *mapkkk3* mutant background and the normal frequencies of *yda* heterozygotes in both backgrounds suggest: (1) a defect in embryogenesis in *yda* homozygotes; and (2) *mapkkk3* has minimal involvement in the process. The absence of *yda* homozygous seedlings in either the *mapkkk5* or *mapkkk3 mapkkk5* backgrounds suggests embryo lethality or a complete failure of male or female gamete transmission. The latter was ruled out based on the reciprocal crosses detailed later. In addition, the reduced frequency of heterozygous *yda* progenies in both *mapkkk5* and *mapkkk3 mapkkk5* backgrounds suggests reduced transmission of either the male and/or female gametes during the reproduction process, that is, *MAPKKK5* and *YDA* play overlapping functions in both gamete transmission and embryogenesis.

Role of *YDA* and *MAPKKK5* in gamete transmission

The above findings suggest a potential defect in male and/or female gamete transmission. The existence of *mapkkk5 yda*/+ and *mapkkk3 mapkkk5 yda*/+ progenies also supports that at least some of the male/female gametes are functional. To investigate this further, we performed reciprocal crosses between *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, or *mapkkk3 mapkkk5 yda*/+ plants and Col-0 wild-type. As shown in Table 2, both male and female *yda* and *mapkkk3 yda* gametes were transmitted at a normal frequency (~50%), suggesting that the reduced homozygous *yda* and *mapkkk3 yda* progenies are a result of defective embryo development. In contrast, both male and female *mapkkk5 yda* and *mapkkk3 mapkkk5 yda* gametes were transmitted at reduced rates (Table 2). We then calculated that the theoretical percentage of homozygous progenies should be at 15.6% and 13.7% for the *mapkkk5 yda* double and *mapkkk3 mapkkk5 yda* triple mutants, respectively, based on the gamete transmission rates. Hence, the absence of viable double and triple homozygous progenies suggests embryo lethality. Since *mapkkk5 yda* and *mapkkk3 mapkkk5 yda* have similar frequencies in gamete transmission and both had complete embryo lethality, we conclude that *MAPKKK3* plays minimal roles in these processes, and that *MAPKKK5* and *YDA* function redundantly in both male and female gamete transmission and embryo development. We can also conclude that *YDA* plays a more important role in embryogenesis since the *yda* single mutant, but not the

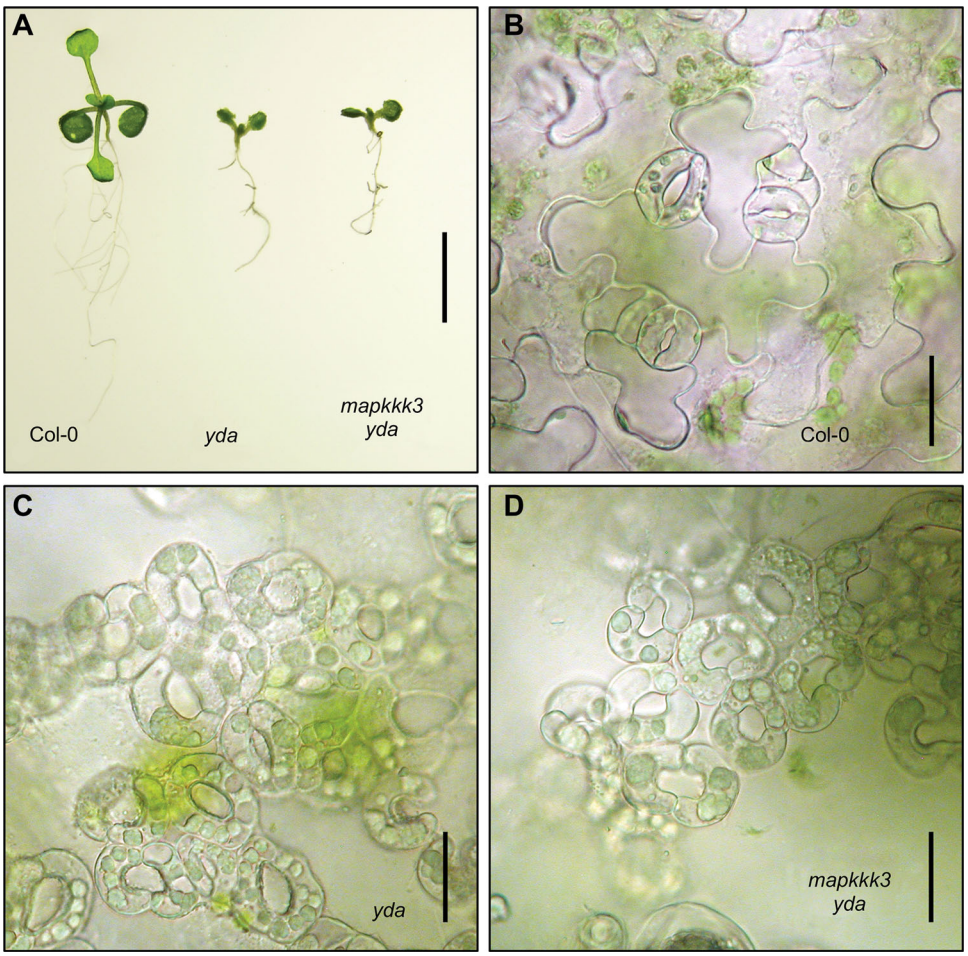

Figure 1. Phenotypes of *yda* single and *mapkkk3 yda* double mutant seedlings
(**A**) Dwarf phenotype of homozygous *yda* single and *mapkkk3 yda* double mutant seedlings. Fourteen-d-old seedlings from progenies of *yda*/+ single and *mapkkk3 yda*/+ double plants were imaged. The genotypes were confirmed by polymerase chain reaction and caps markers. Size bar: 1 cm. (**B**–**D**) The stomatal patterning of Col-0 (**B**), *yda* (**C**), and *mapkkk3 yda* (**D**) seedlings was observed under an Olympus microscope with a digital camera. Size bars: 25 μm.

Table 1. Segregation ratios of *YDA* gene in the progenies of *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ plants

Genotypes	Total	*YDA*	*yda*/+	*yda*
yda/+	106	24.5%	62.3%	13.2%
mapkkk3 yda/+	104	27.9%	60.5%	11.5%
mapkkk5 yda/+	101	42.6%	57.4%	0.0%
mapkkk3 mapkkk5 yda/+	206	45.4%	54.6%	0.0%

Seeds collected from *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ plants were sterilized using bleach and sown on Murashige and Skoog plates after imbibition at 4°C for 3 d. Fourteen-d-old seedlings were collected for polymerase chain reaction genotyping.

mapkkk5 single mutant, has an embryo development defect. In contrast, the *yda* single mutant does not show a defect in gamete transmission, suggesting *MAKKK5* and *YDA* may contribute equally to the process.

Role of *YDA* and *MAPKKK5* in embryogenesis

To examine embryo development, we dissected siliques from Col-0, *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ plants. As shown in Figure 2A, abnormal (shriveled or empty) seeds were observed in all genotypes except Col-0 at significantly higher frequencies (Figure 2B). In siliques from *yda*/+ and *mapkkk3 yda*/+ plants, seeds showed varying degrees of shrinkage. In some seeds, the embryos protruded out from the seed coat (Figure 2A, C), a phenotype also observed in *mpk6* and *mkk4 mkk5* single/double mutants (Zhang et al., 2017). In siliques from *mapkkk5 yda*/+ and *mapkkk3 mapkkk5 yda*/+ plants, empty seeds, an indication of aborted embryogenesis, were observed (Figure 2A, C). In addition, aborted ovules, shown as small remnant placenta attached to the septum of the siliques, were present in the siliques of *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ plants. The observation of aborted ovules is consistent with the reduced female transmission based on the reciprocal cross (Table 2). In contrast to the shriveled seeds observed in

Table 2. Transmission rates of the *yda* mutant gamete in different backgrounds (wild-type, *mapkkk3*, *mapkkk5*, or *mapkkk3 mapkkk5*) based on reciprocal crosses

Genotype (female × male)	Genotype of F1 progenies		TE of *yda* gamete (%)
	yda/+	*YDA*	
Col-0 ♀ × *yda*/+ ♂	64	70	47.8
yda/+ ♀ × Col-0 ♂	83	67	55.3
Col-0 ♀ × *mapkkk3 yda*/+ ♂	67	76	46.9
mapkkk3 yda/+ ♀ × Col-0 ♂	66	60	52.4
Col-0 ♀ × *mapkkk5 yda*/+ ♂	58	98	37.2
mapkkk5 yda/+ ♀ × Col-0 ♂	62	86	41.9
Col-0 ♀ × *mapkkk3 mapkkk5 yda*/+ ♂	50	89	36.0
mapkkk3 mapkkk5 yda/+ ♀ × Col-0 ♂	54	88	38.0

Plants with *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, and *mapkkk3 mapkkk5 yda*/+ genotypes were crossed with Col-0 wild-type as either male or female. After bleach sterilization and imbibition at 4°C for 3 d, the F1 seeds were sown on Murashige and Skoog plates. Fourteen-d-old seedlings were collected for polymerase chain reaction genotyping. TE, transmission efficiency.

the siliques of *yda*/+ and *mapkkk3 yda*/+ plants, mutation of *MAPKKK5* in either the *yda*/+ or *mapkkk3 yda*/+ background lead to only empty seeds, suggesting that MAPKKK5 plays an important role in embryogenesis. Furthermore, similar frequencies of aborted seeds were observed in *mapkkk5 yda*/+ or *mapkkk3 mapkkk5 yda*/+ plants, suggesting that MAPKKK3 plays a minimal role in the process.

Next, we cleared developing siliques collected from plants of all available genotypes and observed embryos at different stages. At the eight-cell stage, *yda* and *mapkkk3 yda* embryos showed suspensors of varying lengths, with some of an adequate length to keep the embryo proper away from the micropyle and toward the center of the endosperm; the embryo proper showed a developmental pattern closely resembling the wild-type (Figure 3). In contrast, all the *mapkkk5 yda* and *mapkkk3 mapkkk5 yda* embryos had extremely short suspensors, which resulted in the embryos residing in the micropylar opening and being constrained by it. At the globular stage, some *yda* and *mapkkk3 yda* embryos showed a relatively normal developmental pattern and were very close to the micropylar opening. As such, this class of embryos might be ones eventually forced out of the seed coat to form the seeds with exposed embryos as shown in Figure 2C. At the globular stage, siliques from *mapkkk5 yda*/+ and *mapkkk3 mapkkk5 yda*/+ plants had either normal-looking seeds (*YDA* or *yda*/+ genotype in either a *mapkkk5* or

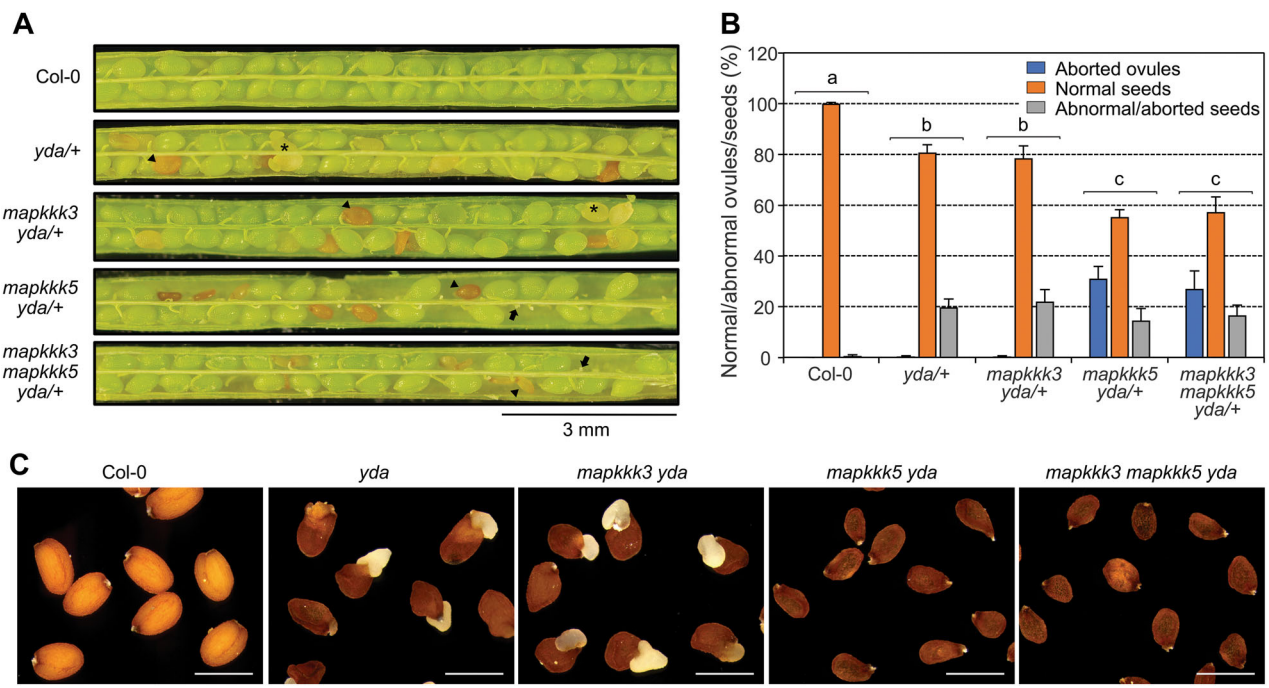

Figure 2. Aborted/abnormal seeds and ovules in the single, double, and triple *mapkkk* mutants

(**A**) Siliques with embryos matured beyond the bend-cotyledon stage were split open from the side to reveal the seeds inside. Representative abnormal/aborted seeds are indicated by arrowheads, aborted ovules by arrows, and seeds with exposed embryos by asterisks. Size bar: 3 mm. (**B**) Aborted ovules, normal seeds, and abnormal/aborted seeds in each silique were counted, and their percentages calculated. Two-way analysis of variance with Tukey's post-hoc test was performed to determine if the differences were significant ($n \geq 6$). Different lower-case letters indicate significant differences among different genotypes ($P < 0.01$). (**C**) Shriveled/exposed or aborted seeds were collected and imaged under a dissecting microscope with a digital camera system. Size bars: 0.5 mm.

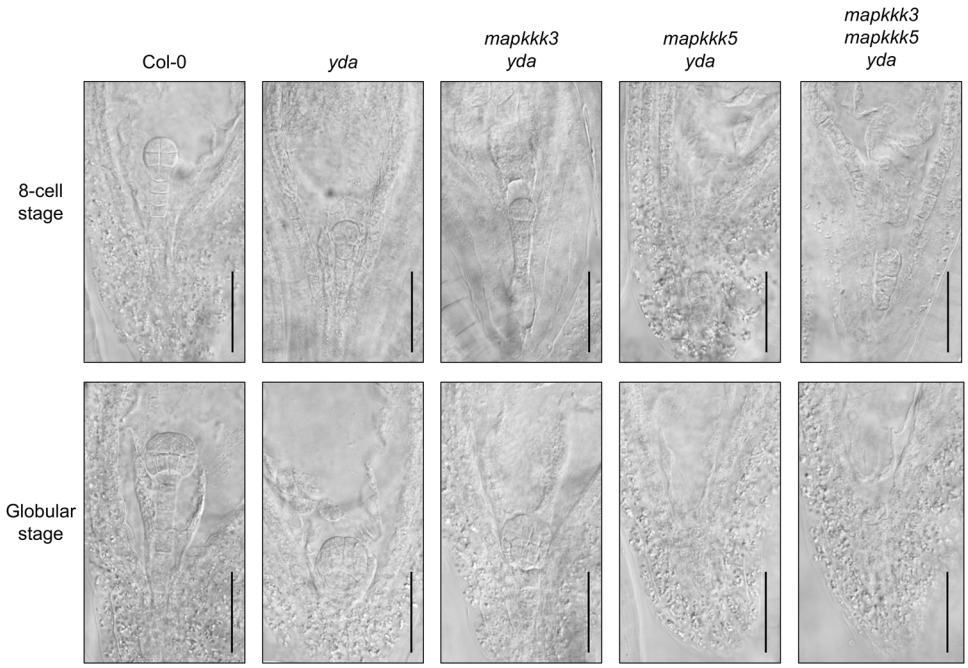

Figure 3. Defective embryo development of *yda*, *mapkkk3 yda*, *mapkkk5 yda*, and *mapkkk3 mapkkk5 yda* mutants
Siliques with embryos at the eight-cell and globular stages were collected from *yda*/+, *mapkkk3 yda*/+, *mapkkk5 yda*/+, or *mapkkk3 mapkkk5 yda*/+ plants. After clearing, the embryos were imaged with differential interference contrast on a Leica Microscope. Size bars: 50 μm.

mapkkk3 mapkkk5 background) or empty seed coats with aborted *yda* homozygous embryos in either the *mapkkk5* or *mapkkk3 mapkkk5* background. A careful examination of the seeds showed remnants of the embryos in the micropylar opening of the seed coat. In the subsequent stage, only empty seed coats were observed, and these eventually changed to a brown color as shown in Figure 2A, C. These observations reveal that all *mapkkk5 yda* and *mapkkk3 mapkkk5 yda* embryos are aborted after the globular stage, while some *yda* and *mapkkk3 yda* embryos can develop further and form seeds. This is consistent with the observation of reduced homozygous seedlings in the progenies of *yda*/+ and *mapkkk3 yda*/+ plants and the failure to recover *mapkkk5 yda* and *mapkkk3 mapkkk5 yda* mutant plants.

Generation of weak *yda* deletion alleles using CRISPR-Cas9

Because of the essential functions of *MAPKKK5* and *YDA* in embryogenesis, we were unable to obtain triple knockout mutants for the analysis of their function(s) in plant immunity. We then turned to the idea of generating weak *yda* deletion alleles using CRISPR-Cas9. Two sites in the first exon of the *YDA* gene (Figure 4A), which encodes the non-kinase domain of the *YDA* gene, were targeted in the *mapkkk3 mapkkk5* double mutant background using the pYAO CRISPR-Cas9 system (Yan et al., 2015). Screening of T1 plants using a pair of primers that flank the two CRISPR-Cas9 target sites allowed the identification of deletion lines. They were then backcrossed to *mapkkk3 mapkkk5* plants to remove the Cas9 gene. Sequencing of the region flanked by the two target sites allowed the identification of multiple in-frame deletion mutant alleles (*yda-del* mutant alleles, Figure 4A). However, no frameshifting mutant allele with loss-of-function *yda* was identified. Representative genotyping gel image and sequencing identification of the mutants are shown in Figure S3. We then selected an allele with 42-AA (amino acid) deletion, named *yda-Δ42*, for further analysis. The triple mutant plants (genotype: *mapkkk3 mapkkk5 yda-Δ42*) had a smaller stature in comparison to the *mapkkk3 mapkkk5* double mutant and Col-0 wild-type (Figure 4B). It also had a stomata clustering phenotype, although much less severe than that of the *yda* knockout mutant (Figure 4C).

CRISPR-Cas9 deletion mutant of *YDA* further compromises the immunity of *mapkkk3 mapkkk5* double mutant

To test the activation of MPK3 and MPK6 in the *mapkkk3 mapkkk5 yda-Δ42* triple mutant in the defense response, we treated seedlings with flg22 for various times and collected samples to determine the phosphorylation activation of MPK3 and MPK6 in Col-0, *mapkkk3 mapkkk5* double mutant, and *mapkkk3 mapkkk5 yda-Δ42* triple mutant. As shown in Figure 5A, partial loss of *YDA* function in the double *mapkkk3 mapkkk5* mutant background further reduced the phosphorylation activation of MPK3/MPK6 in response to flg22, suggesting that YDA functions redundantly with MAPKKK3 and MAPKKK5 in the process. Previously, it was shown that the double *mapkkk3 mapkkk5* mutant is more susceptible to *Pseudomonas syringae pv. tomato DC3000* (*Pst*). When we compared *Pst* growth in Col-0, *mapkkk3 mapkkk5*, and *mapkkk3 mapkkk5 yda-Δ42* plants, we observed that the

partial loss of *YDA* function further compromised plant resistance against *Pst* (Figure 5B). This again demonstrated that YDA plays an important role in plant immunity together with MAPKKK3 and MAPKKK5.

DISCUSSION

Arabidopsis MPK3/MPK6 and their upstream MAPKKs, MKK4 and MKK5, play important roles in plant immunity and growth/development. YDA has been shown to be the MAPKKK upstream of MKK4/MKK5-MPK3/MPK6 to form a complete MAPK cascade in plant growth and development, while MAPKKK3/MAPKKK5 function upstream of MKK4/MKK5-MPK3/MPK6 in plant immunity (reviewed in Sun and Zhang, 2022; Zhang and Zhang, 2022). In this report, we demonstrated that YDA is also involved in plant immunity together with MAPKKK3/MAPKKK5. In addition, MAPKKK3/MAPKKK5, especially MAPKKK5, also play critical roles in plant growth and development, together with YDA. These findings could explain why: (1) there is only a partial loss of MPK3/MPK6 activation in the *mapkkk3 mapkkk5* double mutant in response to PAMP treatment (Figure S2; Bi et al., 2018; Sun et al., 2018); and (2) the *yda* knockout mutant has weaker growth and developmental phenotypes than the *mpk3 mpk6* double mutant (Wang et al., 2007). For instance, no *mpk3 mpk6* double mutant progeny were recovered from either *mpk3 mpk6*/+ or *mpk3*/+ *mpk6* plants, while *yda* homozygotes were recoverable as severely dwarfed plants (Figure 1A; Lukowitz et al., 2004; Wang et al., 2007). At this stage, we cannot test the defense response of the triple MAPKKK knockout mutant because of embryo lethality. However, based on the further reduction of (1) MPK3/MPK6 activation in response to flg22 treatment, and (2) *Pst* resistance in the *mapkkk3 mapkkk5 yda-Δ42* triple mutant in comparison with the *mapkkk3 mapkkk5* double mutant (Figure 5), we can conclude that *YDA* also plays an important role in plant immunity.

The overlapping, but somewhat differential, functions of *YDA*, *MAPKKK3*, and *MAPKKK5* in plant immunity and growth/development are likely a result of their differential expression patterns. The amount of MAPKKK protein present in a specific type of cell/tissue/organ could determine its contribution to the signaling strength in a specific biological process. Based on the Arabidopsis Atlas eFP Browser on bar.utoronto.ca website (Klepikova et al., 2016), all three MAPKKKs are expressed in leaves at comparable levels, making it possible for all three to contribute to plant immune signaling. In contrast, *MAPKKK5* and

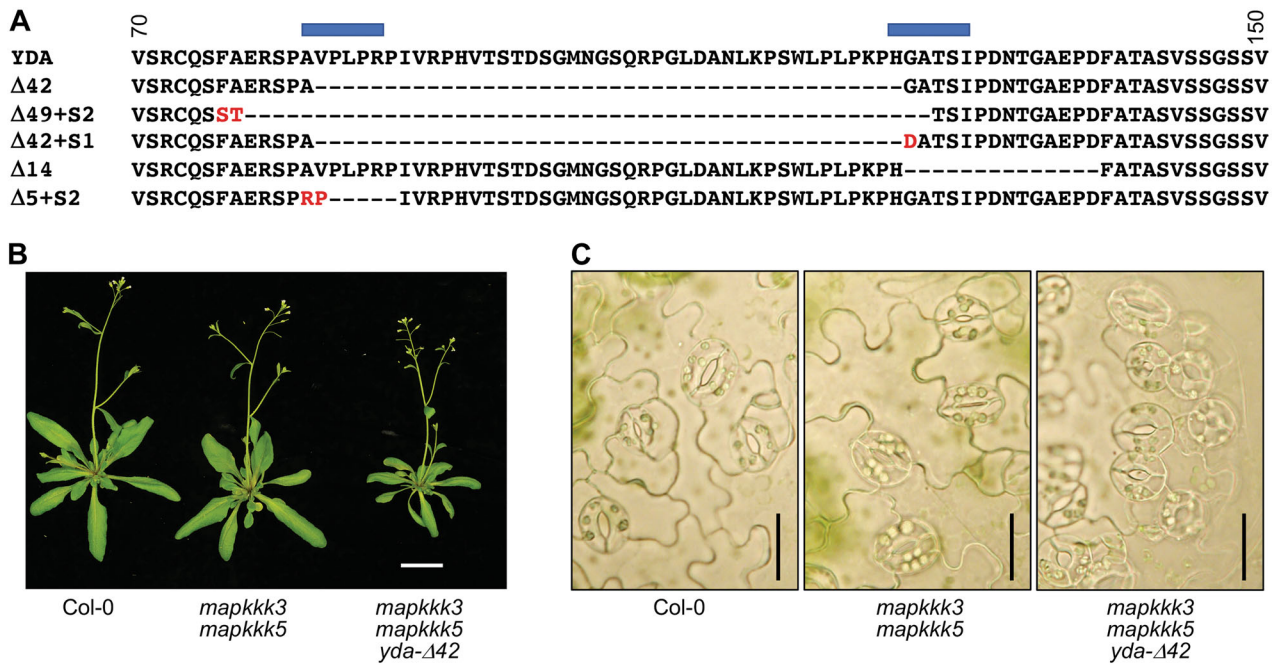

Figure 4. Weak *yda* mutant alleles generated using clustered regularly interspaced short palindromic repeats (CRISPR) – CRISPR-associated protein 9 (Cas9) in a *mapkkk3 mapkkk5* background have weak *yda* phenotype

(**A**) A CRISPR-Cas9 construct containing two single-guide RNAs (sgRNAs) targeting two different sites in the N-terminal region of *YDA* was used to generate deletion *yda* mutant alleles in the *mapkkk3 mapkkk5* double mutant background. Polymerase chain reaction (PCR) genotyping was used to identify deletion mutant alleles and subsequent sequencing of PCR fragments revealed the nature of these mutations. Translated amino acid sequences were aligned to the wild-type YDA sequence. Blue bars above the sequence indicate the corresponding position of the two sgRNA target sites. Numbers indicate the beginning and ending amino acid (AA) positions of the wild-type YDA protein. All mutant alleles identified are in-frame deletion alleles (*yda-del*). Some had substitutions of a few AAs (marked in red color). Mutant alleles with a 42-AA deletion (*yda-Δ42*) were the most common and were used for experiments. **(B)** Dwarf stature of the *yda-Δ42* mutant plants. Four-week-old Col-0, *mapkkk3 mapkkk5*, and *mapkkk3 mapkkk5 yda-Δ42* plants grown under 14 h light : 10 h dark cycle was imaged. Size bar: 2 cm. (**C**) Stomatal clustering in the *yda-Δ42* mutant. The epidermis of 12-d-old Col-0, *mapkkk3 mapkkk5*, or *mapkkk3 mapkkk5 yda-Δ42* seedlings was observed. Size bars: 25 μm.

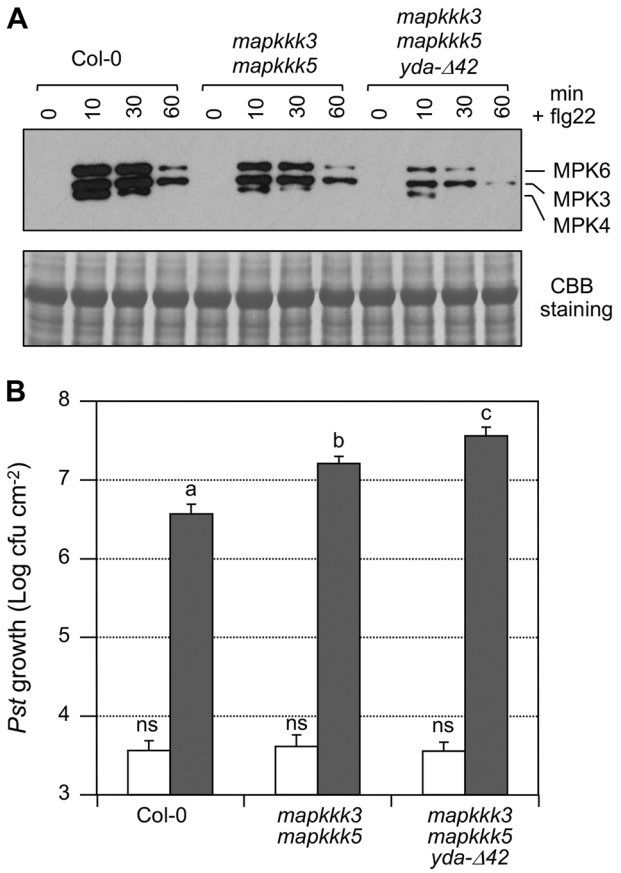

Figure 5. Compromised *MITOGEN-ACTIVATED KINASE3 (MPK3)/MPK6* activation and pathogen resistance in the *mapkkk3 mapkkk5 yda-Δ42* triple mutant

(**A**) MPK3/MPK6 activation triggered by flg22 is further reduced in the *mapkkk3 mapkkk5 yda-Δ42* triple mutant seedlings. Fourteen-d-old Col-0, *mapkkk3 mapkkk5* double, and *mapkkk3 mapkkk5 yda-Δ42* triple mutant seedlings were treated with flg22 (30 nmol/L final concentration) and collected at the indicated time. The phosphorylation activation of MPK3 and MPK6 were detected by using anti-pTEpY antibody. An equal amount of total protein (10 μg) was loaded in each lane, as confirmed by Coomassie brilliant blue staining of duplicate gels. (**B**) Four-week-old Col-0, *mapkkk3 mapkkk5*, and *mapkkk3 mapkkk5 yda-Δ42* plants were infiltration-inoculated with *Pseudomonas syringae pv. tomato DC3000* (*Pst*) (optical density at 600 nm = 0.0005). Inoculated amount and bacterial growth were measured at 0 and 3 d post-inoculation, respectively. Values are means ± *SD*, $n = 5$. Lower-case letters above the bars indicate significantly different groups (one-way analysis of variance, $P < 0.01$).

YDA are expressed at much higher levels in flowers than *MAPKKK3*, which could explain why the mutation of *MAPKKK3* showed little impact on plant reproduction, including embryogenesis (Figures 2, 3; Table 1) and gamete transmission (Table 2) in the *yda* mutant background. Further, *mapkkk3 yda* double mutant seedlings were phenotypically identical to *yda* single mutant seedlings (Figure 1). In contrast, both *MAPKKK5* and *YDA* contribute to the signaling process during embryogenesis and gamete transmission, resulting in the failure to recover the homozygous *mappkkk5 yda* double or *mapkkk3 mapkkk5 yda* triple mutants.

A partial loss of *YDA* function in *yda-Δ42* mutants in the *mapkkk3 mapkkk5* background had a major impact on the activation of downstream MPK3/MPK6 in response to flg22 treatment (Figure 5A), suggesting YDA plays an equally important function as MAPKKK3 and MAPKKK5 in MPK3/MPK6 activation in plant immunity. Single mutants of all three genes showed little impact on the activation of MPK3/MPK6 (Figure S2; Bi et al., 2018; Sun et al., 2018). In the double combinations, the activation of MPK3/MPK6 was not decreased in *mapkkk3 yda* but was partially reduced in *mapkkk3 mapkkk5* (Figure S2; Bi et al., 2018; Sun et al., 2018). We were unable to test the *mapkkk5 yda* double or *mapkkk3 mapkkk5 yda* triple knockout plants because of their embryo lethality. MPK3/MPK6 activation is very rapid in response to PAMP treatment, suggesting the pre-existence of a protein complex in MAPK signaling. At this stage, the factor(s) involved in the formation of this putative complex in plant MAPK signaling is largely unknown. Because of the presence of large extensions in the N- and/or C-termini of the MAPKKKs, there is a possibility that they function as scaffolds to hold MAPKK(s) and MAPK(s) in the MAPK cascade together as in the mammalian MEKK1, also a large protein with binding sites for other components of the MAPK cascade (Pearson et al., 2001). The kinase domain of YDA resides in the middle of the protein (AA residues 400 to 656 out of the 883 total amino acids). Small deletions in the first 150-AA region (Figure 4A) is unlikely to affect the activity of the kinase domain directly. Further reduction of MPK3/MPK6 activation observed in the *mapkkk3 mapkkk5 yda-Δ42* triple mutant (Figure 5A) is likely a result of a reduced functionality of the non-kinase domain of YDA in the MAPK cascade, for instance in its interaction with either upstream components, such as receptor-like cytoplasmic protein kinases or downstream MAPKKs/MAPKs. Further research is needed to define the functional domains of this large MAPKKK in *Arabidopsis*.

It is possible that, when one or two MAPKKK genes are mutated, the remaining member(s) can maintain a complex with MAPKK(s) and MAPK(s) to sustain a normal or close-to-normal activation of MPK3/MPK6 and the downstream signaling process. In this scenario, compromised activation of downstream MAPK(s) occur only when the total amount of MAPKKK protein drops below a threshold needed to maintain the signaling strength. In the various biological processes, these three MAPKKKs may have differential contributions because of their differential expression patterns. With respect to plant immunity, *mapkkk3 mapkkk5* double mutant starts to show compromised MPK3/MPK6 activation and disease resistance, and partial loss of *yda* function in the *mapkkk3 mapkkk5* background (Figure 5) leads to further reduction in the plant immune response. It is likely that triple *mapkkk3 mapkkk5 yda* knockout mutation might have no MPK3/MPK6 activation after PAMP treatment. However, we cannot test this at this stage because of the embryo lethality. In plant embryogenesis, the *yda* single mutant leads to severe phenotype, but is still viable. In contrast, the loss of *mapkkk5* on top of *yda* results in complete failure of embryogenesis. In this process, *YDA* plays a more important role than *MAPKKK5* because single *mapkkk5* mutant does not have embryogenesis defect.

However, in male/female gamete transmission, neither *yda* nor *mapkkk5* mutant has a phenotype, but the double mutant gametes show reduced transmission (Table 2), suggesting that YDA and MAPKKK5 might contribute equally to the process.

YDA was first identified as a MAPKKK involved in embryogenesis and stomatal differentiation (Bergmann et al., 2004; Lukowitz et al., 2004). Later, YDA was placed upstream of the MKK4/MKK5–MPK3/MPK6 module in a variety of growth/developmental processes, including stomatal differentiation, embryogenesis, inflorescence architecture, and root development (Wang et al., 2007; Bayer et al., 2009; Meng et al., 2012; Smekalova et al., 2014; Ueda et al., 2017; Lu et al., 2020; Shao et al., 2020). This MAPK cascade (YDA–MKK4/MPK5–MPK3/MPK6) is a key signaling module downstream of ERECTA (ER) and ER-like (ERL) receptors in plant growth and development (reviewed in Sun and Zhang, 2022; Zhang and Zhang, 2022). Recently, several studies have implicated *YDA* in plant immunity but with contradictory results. It was reported that plant resistance to pathogens was compromised in weak *yda* mutant alleles, and that plants expressing the constitutively active YDA protein showed broad-spectrum resistance to fungi, bacteria, and oomycetes with different colonization modes (Sopena-Torres et al., 2018). Furthermore, ER/ERL receptors are upstream of the YDA–MKK4/MKK5–MPK3/MPK6 MAPK cascade in a shared signaling pathway in plant immunity and stomatal formation. Tomato orthologs of *Arabidopsis YDA* were also shown to play a positive role in disease resistance (Tellez et al., 2020). However, in another study using RNA interference suppression of *YDA*, it was concluded that *YDA* and *MAPKKK3/MAPKKK5* interact antagonistically in plant development and immunity (Sun et al., 2018). The developmental defects caused by the silencing of *YDA* were suppressed in the double *mapkkk3 mapkkk5* mutant. In addition, *YDA* gene silencing enhanced the activation of MPK3 and MPK6 after PAMP treatment, suggesting a negative role for *YDA* in the plant immune response.

Our conclusion in this report is that YDA, MAPKKK3, and MAPKKK5 have overlapping functions in both plant immunity and growth/development. All three MAPKKKs function as positive regulators upstream of MPK3/MPK6 in the same MAPK cascade (Figure 6). It is likely that they contribute differentially to the activation of MPK3/MPK6 and the downstream events in different biological processes, dependent on their expression levels in specific cells/tissues/organs. MPK3 and MPK6 have been shown to be downstream of a variety of plant receptors/sensors in plant growth/development and immunity (reviewed in Sun and Zhang, 2022; Zhang and Zhang, 2022). The sensing of either external cues or internally produced ligands by these receptors leads to the activation of MPK3 and MPK6 through the upstream MKK4/MKK5 MAPKKs and YDA/MAPKKK3/MAPKKK5 MAPKKKs, which in turn activates events/responses further downstream in plant growth/development and immunity.

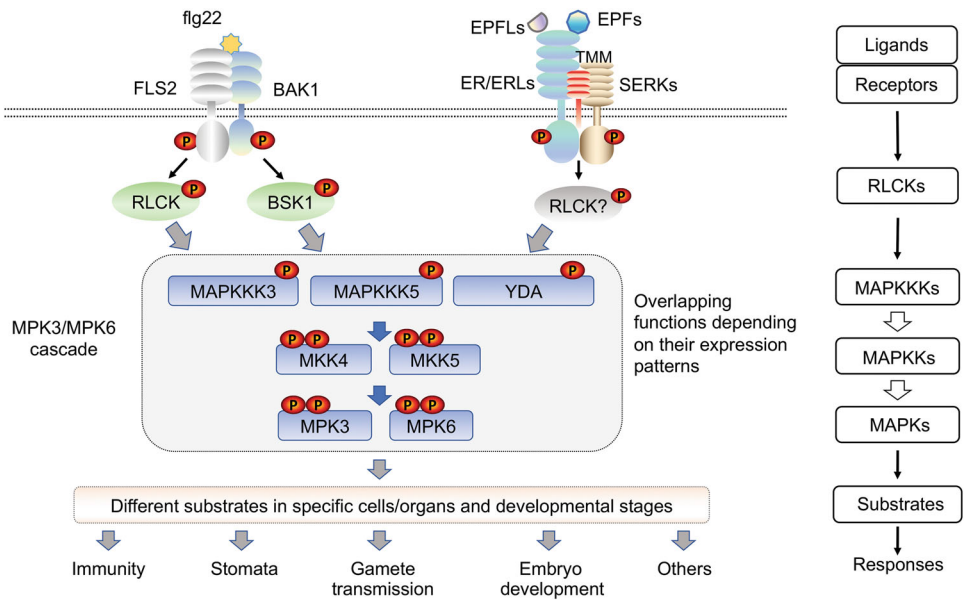

Figure 6. Overlapping functions of YDA, MAPKKK3, and MAPKKK5 in the MPK3/MPK6 MAPK cascade in signaling plant immunity and growth/development

Plant perception of either exogenously derived pathogen-associated molecular patterns such as flg22 or endogenously produced peptide ligands such as epidermal factors (EPFs) and EPF-likes (EPFLs) by plant pattern-recognition receptors (PRRs, such as FLS2) and other receptor-like protein kinase receptors such as ERECTA (ER) and ER-likes (ERLs) activate the MPK3/MPK6 MAPK cascade. MKK4 and MKK5, two redundant MAPKKs, function upstream of MPK3/MPK6. Three MAPKKKs including YDA, MAPKKK3, and MAPKKK5 play overlapping, yet differential, functions in the MPK3/MPK6 cascade. Depending on the levels of their expression in different cells/tissues/organs, they show differential functions in plant immunity and growth/development upstream of MKK4/MKK5–MPK3/MPK6 in a variety of biological processes.

MATERIALS AND METHODS

Plant materials and growth conditions

Mutant and wild-type plants of the *Arabidopsis thaliana* Columbia (Col-0) ecotype were used in all experiments. A T-DNA insertion mutant of *YDA* was obtained from the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu; SALK_105078; Alonso et al., 2003) and was previously described (Wang et al., 2007). The double *mapkkk3-2 mapkkk5-2* mutant was kindly provided by Dr. Jian-Min Zhou (Bi et al., 2018). Seeds were plated on half-strength Murashige and Skoog medium with 0.45% PhytoAgar after surface sterilization and imbibing at 4°C for 3 d. Plates were incubated in a tissue culture chamber at 22°C under continuous light (50 μE/m^2/s) for 5–7 d. Seedlings were then transplanted into soil and grown in a growth chamber with a 14-h light/10-h dark cycle (100 μE/m^2/s) unless stated otherwise.

Generation of *yda* deletion mutant alleles using CRISPR-Cas9

The CRISPR/Cas9 construct was prepared by inserting two *YDA* single-guide RNA (sgRNA) into a pYAO:hSpCas9 vector as described previously (Yan et al., 2015). After transformation into the *mapkkk3-2 mapkkk5-2* double mutant plants (Bi et al., 2018), T1 *yda* deletion mutants in *mapkkk3 mapkkk5* background were identified by polymerase chain reaction (PCR) genotyping. Cas9-free T3 homozygous mutant individuals were identified, and the T4 generation was used for experiments.

Observation of embryos, seeds, and stomata

For Nomarski microscopy of cleared seeds, siliques with embryos at the eight-cell and globular stages were collected from flowering plants and cleared for 2 h in 0.5 mL of clearing solution (Herr, 1971). Cleared siliques were examined using a Leica (Wetzlar, Germany) DM 5500B microscope equipped with Nomarski optics. Siliques with seeds after the bent-cotyledon stage were dissected and imaged using a Panasonic digital camera. Defective seeds at the maturation stage were selected under a dissecting microscope and imaged using a Leica M205 FA stereomicroscope. Stomata on the leaf surface were observed and imaged using an Olympus (Tokyo, Japan) microscope with a digital camera attachment.

Protein extraction and immunoblot analysis

Protein extraction and immunoblot were carried out as previously described (Su et al., 2018). Total proteins (10 μg) were separated on 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis. For better separation, electrophoresis was continued for another 15 min after the blue tracking dye came out of the gel. Phosphorylation activation of MPK3 and MPK6 was detected by using anti-pTEpY (Cell Signaling Technology, Danvers, MA, USA), dilution 1:5 000). After incubation with primary antibodies and washing, the blots were incubated with horseradish peroxidase-conjugated goat-anti-rabbit immunoglobulin G secondary antibodies (Sigma, St. Louis, MO, USA, dilution 1:10,000), and the bands were visualized using an enhanced chemiluminescence kit (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions.

Pathogen inoculation and disease resistance assay

Pseudomonas syringae pv. tomato (*Pst*) *DC3000* inoculation and disease resistance assays were performed as previously described (Su et al., 2018). *Pst* was grown overnight at 28°C on Pseudomonas Agar (Difco Laboratories, Detroit, MI, USA) with Rif (50 μg/mL). Four-week-old Col-0 and mutant plants grown under a short-day light cycle (10 h of light and 14 h of dark) were infiltrated with *Pst* (optical density at 600 nm = 0.0005 in 10 mmol/L $MgCl_2$). Pathogen growth was determined 3 d post-inoculation.

Quantification and statistical analysis

At least three independent repetitions were performed. Data from one of the independent repetitions with similar results are shown in the figures. Statistical analysis of the experiments is detailed in the figure legends. GraphPad Prism (https://graphpad.com) was used for statistical analyses. One-way analysis of variance (ANOVA) or two-way ANOVA analysis with Tukey's post-hoc test was performed to evaluate whether the differences were statistically significant. Lower-case letters above the columns were used to indicate differences that are statistically significant with *P*-values indicated in figure legends.

Accession numbers

Sequence data from this article can be found in The Arabidopsis Information Resource database (https://www.arabidopsis.org) under the following accession numbers: AT1G63700 (YDA or MAPKKK4), AT1G53570 (MAPKKK3 or MAPKKKα), and AT5G66850 (MAPKKK5).

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CONFLICTS OF INTEREST

The authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS

S.Z. and Y.L. designed the project. Y.L., E.L., O.S., R.F.B., and S.Z. performed the experiments. Y.L. and S.Z. analyzed

the results and wrote the manuscript. All authors read and approved of this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.13309/suppinfo

Figure S1. Phylogenetic analysis of the MEKK subgroup of all putative *Arabidopsis* mitogen-activated protein kinase kinase kinases (MAPKKKs)

Figure S2. Genotyping and sequencing identification of *yda-Δ42* mutant allele generated using clustered regularly interspaced short palindromic repeats (CRISPR) – CRISPR-associated protein 9 (Cas9)

Figure S3. Activation of mitogen protein kinase (MPK)3/MPK6 in various *mapkkk* mutant seedlings after flg22 treatment

Table S1. Primers used in this study