Running head:

Carotenoid regulation of root growth

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Full title:

Carotenoid metabolism negatively regulates auxin-mediated root growth

One-sentence summary

The β-carotene-derived apocarotenoid retinal negatively regulates auxin levels and signaling and controls root growth.

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Abstract

Carotenoids support plant developmental activities as photosynthesis and photoprotective pigments, they also provide precursors for production of phytohormone abscisic acid and strigolactones, or bioactive apocarotenoids. Herein, the role of carotenoids as sources of apocarotenoids, which control endogenous auxin levels and plant root architecture was examined. Inhibition of β-carotene biosynthesis with the phytoene desaturase (PDS) inhibitor fluridone suppresses root growth, consequently inducing lateral root growth. PDS was confirmed to be the target of fluridone via the expression of the fluridone-insensitive trait *35S::mHvPDS* in an *Arabidopsis* plant. Inhibiting β-carotene biosynthesis elevated endogenous auxin levels and activated auxin signaling, thereby suppressing root growth. In addition, the root growth of the auxin-deficient mutant *yucQ* was partially restored via fluridone treatment. Consequently, suppressing PDS activity increases endogenous auxin levels and suppresses root growth. Conversely, the carotenoid pool indirectly suppresses endogenous auxin levels. Exogenous application of retinal, an apocarotenoid derived from β-carotene, complemented fluridone-mediated root growth inhibition and partially recovered auxin-mediated growth inhibition. Moreover, retinal inhibited the induction of the auxin-inducible reporter *pIAA19::ElucPEST*, indicating an antagonistic effect of retinal on endogenous auxin levels. Interestingly, the auxin-deficient mutants *wei2 wei7* and *yucQ* accumulated more β-carotene compared with the wild type. The expression of *CCDs*, which converts β-carotene into apocarotenoids, is auxin-inducible and increases following fluridone treatment. These results indicate feedback regulation during apocarotenoid biosynthesis in plant tissue. Thus, we conclude carotenoid regulates auxin levels and response, and this regulation is mediated by apocarotenoid retinal.

Introduction

The root system plays a vital role in water and nutrient uptake, anchors plants to the soil, and is a source of signaling chemicals that travel from root to root and root to shoot. The phytohormone auxin participates in establishing the root system architecture, which controls

root growth and lateral root patterning (Fukaki and Tasaka, 2009; Moreno-Risueno et al., 2010). Auxin signaling begins with the degradation of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins through ubiquitination by the E3 ligase complex TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN ($SCF^{TIR1/AFB}$) (Dharmasiri et al., 2005). The functional repression of the transcription factor AUXIN RESPONSE FACTOR (ARF) by Aux/IAA is inactivated incrementally by cellular auxin levels (Leyser, 2002; Shimizu-Mitao and Kakimoto, 2014). A gain-of-function mutation of typical *aux/iaa* exhibited insensitivity to exogenous auxin addition and was accompanied by a lateral root (LR) defect (i.e., *massugu2/auxin/indole-3-acetic acid19* [*msg2/aux/iaa19*], *solitary root/auxin/indole-3-acetic acid14* [*slr/aux/iaa14*], and *suppressor of hy2 mutation 2/auxin/indole-3-acetic acid3* [*shy2/aux/iaa3*]). The auxin influx carriers AUXIN RESISTANT1 (AUX1) and LIKE AUX1 (LAX) (Swarup and Peret, 2012), auxin efflux carrier PINFORMED (PIN) (Paponov et al., 2005), and the ATP-binding cassette B /MULTIPLE DRUG RESISTANCE/P-GLYCOPROTEIN (ABCB/MDR/PGP) (Geisler and Murphy, 2006) regulate the flow and cellular levels of auxin to generate an auxin gradient, which also shapes the root architecture (Vieten et al., 2007).

The conventional pathway of IAA biosynthesis in *Arabidopsis* involves the conversion of tryptophan to indole-3-pyruvic acid (IPyA) by TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA), followed by oxidation of IPyA to IAA by the activity of YUCCA (YUC) flavin monooxygenases. YUCCA is the rate-limiting enzyme in this pathway and comprises 11 gene families in *Arabidopsis* (Won et al., 2011; Zhao, 2012). *YUCCA* genes are highly redundant (Cheng et al., 2006; Xu et al., 2017) and multiple knockouts of the gene family members result in an auxin-deficient phenotype (Cheng et al., 2007; Chen et al., 2014). However, there are several non-IpyA pathways of IAA biosynthesis, including the indole-3-acetaldoxime (IAOx) pathway. The IAOx pathway generates indole-glucosinolate (IG) that prevent herbivores and also generates IAA through indole-3-acetonitrile (IAN) or indole-3-acetamide (IAM) (Sugawara et al., 2009). One of the cytochrome P450 gene family members of the IAOx pathway, *SUPERROOT2/CYP83B1* (*SUR2*), oxidizes IAOx as a substrate for IG biosynthesis. A loss-of-function mutant of *sur2* accumulates IAA and exhibits

a pleiotropic effect, including high LR density, adventitious root, long hypocotyl, and epinastic cotyledon (Delarue et al., 1998; Barlier et al., 2000; Goto et al., 2023). Chorismic acid is a precursor of tryptophan biosynthesis, where the first step is catalyzed by a heterodimer of anthranilate synthase, WEAK ETHYLENE INSENSITIVE 2/ANTHRANILATE SYNTHASE α1 (WEI2/ASA1), and WEI7/ANTHRANILATE SYNTHASE β1 (WEI7/ASB1). The loss-of-function mutant of the anthranilate synthase subunit, *wei2* or *wei7*, decreases endogenous auxin levels and moderately reduces root length (Stepanova et al., 2005).

Carotenoids are a group of plastidial isoprenoid pigments that mainly synthesized by plants, fungi, algae and bacteria (Sun et al., 2018). In flowering plants, the photopigment carotenoid is synthesized by the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway (Rodriguez-Concepcion and Boronat, 2002). Geranylgeranyl pyrophosphate (GGPP) is condensed to phytoene by PHYTOENE SYNTHASE (PSY), which undergoes desaturation by PHYTOENE DESATURASE (PDS) to form ζ-carotene and is subsequently converted to tetra-*cis*-lycopene by ZETA-CAROTENE DESATURASE (ZDS) (Breitenbach and Sandmann, 2005). Tetra-*cis*-lycopene is isomerized to all-*trans*-lycopene by CAROTENOID CHLOROPLAST REGULATOR2/CAROTENOID ISOMERASE (CCR2/CRTISO) and its expression is mediated by histone methyltransferase SET DOMAIN GROUP 8 (SDG8)/CCR1 (Park et al., 2002; Cazzonelli et al., 2010). Subsequently, the pathway branches to produce α- or β-carotene (**Fig. 1A**). Fluridone is a herbicide used for eliminating aquatic plants and inhibiting PDS activity, resulting in chlorosis in plants (Bartels and Watson, 1978). Carotenoids exhibit diverse functions. They act as a protective photopigment in the photosynthetic complex and membrane, add color to fruit, and are a source of bioactive apocarotenoids, which are the products of oxidative cleavage of carotenoids (Cazzonelli, 2011). Although the apocarotenoid phytohormone abscisic acid (ABA) as well as strigolactones (SLs) (Schwartz et al., 1997; Umehara et al., 2008; Zhang et al., 2010) are well characterized, there has been an increase in the number of new bioactive apocarotenoids discovered during this century (Auldridge et al., 2006; Zheng et al., 2021). For example, β-cyclocitral is recently identified as a root growth regulator and reportedly

exhibits a positive effect on primary root (PR) elongation and LR capacity (Dickinson et al., 2019). Anchorene, a β-carotene-derived regulatory metabolite, promotes anchor root formation by modulating auxin distribution (Jia et al., 2019). Another example is retinal, a precursor of vitamin A and retinoic acid in vertebrates (Olson and Hayaishi, 1965) that it also exists in higher plants (Koschmieder et al., 2021) and plays a role in the periodic branching of the root (Dickinson et al., 2021). In addition to their role in root architecture, the apocarotenoids ionone derivatives repel or attract plant herbivores. The apocarotenoids bixin and safranal are also important in the food industry as color additives (Zheng et al., 2021). The family of carotenoid cleavage dioxygenases (CCD) and 9-*cis* epoxycarotenoid dioxygenase (NCED) are responsible for carotenoid catabolism and producing apocarotenoids. CCD1 and CCD4 exhibit a wide range of cleavage specificities, ranging from phytoene to neoxanthin, to generate apocarotenoids, such as bixin, crocin, safranal, α-ionone, β-ionone, and β-cyclocitral. CCD7 and CCD8 are involved in the synthesis of SLs from β-carotene. NCEDs are responsible for the production of ABA (Yuan et al., 2015).

Carotenoids function as photopigments working with chlorophylls in chloroplasts and are the precursors of apocarotenoids (e.g., retinal) and phytohormones (e.g., ABA and SLs). Auxin modulates chlorophyll content and chloroplast biogenesis in the root (Kobayashi et al., 2012), and regulates carotenoid accumulation during fruit ripening in tomato (Su et al., 2015). Auxin and apocarotenoid SLs can regulate the level of each other and contribute to shoot branching (Hayward et al., 2009). Application of IAA increases ABA sensitivity to seed germination (Liu et al., 2007). Consequently, auxin plays a vital role in regulating plastid activities, controlling carotenoid contents and interacting with apocarotenoids for developmental activities. However, the interaction between auxin and carotenoids remains to be elucidated, especially how carotenoid and apocarotenoid contents affect auxin homeostasis and control root architecture. In this study, we found that carotenoid biosynthesis inhibition resulted in auxin level elevation and response induction in the root. Apocarotenoid retinal rescued carotenoid-mediated and auxin-mediated root growth suppression, suggesting an intermediate position of retinal in carotenoid and auxin interaction pathway. Herein, we demonstrate that carotenoid biosynthesis negatively couples with auxin biosynthesis and that

the exogenous apocarotenoid retinal may be a negative regulator for endogenous auxin levels.

Results

Fluridone suppresses the growth of the primary root

Fluridone inhibited PR growth and induced LR density in a dose-dependent manner (**Fig. 1, B, D and E**). The emerging true leaves of the seedlings were bleached following fluridone treatment; however, the established leaves, such as the cotyledons, were bleached to a lesser extent (**Fig. 1, C and F**). Other PDS inhibitors, including norflurazon, diflufenican, and picolinafen, also suppressed PR growth and increased LR density comparable with the effects of fluridone treatment (Supplemental Fig. S1). To identify the target of fluridone for root growth inhibition, the fluridone-insensitive trait PDS (*35S::mHvPDS*) (Arias et al., 2006) was introduced into the *Arabidopsis* plant. The *35S::mHvPDS* plants exhibited less bleached leaves (**Fig. 1C**) and less reduced carotenoid content (**Fig. 1F**) following fluridone treatment, confirming that mutated *PDS* is less sensitive to fluridone (Arias et al., 2006). The *35S::mHvPDS* plants also exhibited partial resistance to root growth inhibition (**Fig. 1D**). These results indicate that PDS is the target of fluridone for PR growth inhibition and implicates the carotenoid biosynthesis pathway for the involvement to the root growth regulation. PR growth was inhibited for 2 days following fluridone treatment, with the extent of inhibition further increasing after the third day (Supplemental Fig. S3). LR density was observed to increase with fluridone treatment in the wild type (WT) and to a lesser extent, in the *35S::mHvPDS* plants (**Fig. 1E**). Since the LR initiation and emergence are under the control of auxin signaling (Du and Scheres, 2018), increased LR density indicates the activation of auxin signaling.

Fluridone increases endogenous auxin levels and activates auxin signaling

The fluridone-induced PR growth inhibition and LR density elevation prompted us to measure the endogenous auxin levels. Although the WT exhibited increased IAA levels 8 h

and 24 h following fluridone treatment, and then auxin levels returned to normal range (**Fig. 2**; Supplemental Fig. S2A). However, no significant change of auxin levels was observed in the *35S::mHvPDS* plants (Supplemental Fig. S2B). To determine the kinetics of auxin signaling, a time-lapse image of the artificial auxin reporter *DR5:Luciferase* (*DR5:LUC*) (Moreno-Risueno et al., 2010) was captured and compared between the WT and *35S::mHvPDS* plants. Fluridone-induced *DR5:LUC* expression in the root tip and root–shoot junction was observed in the WT and to a lesser extent, in the *35S::mHvPDS* plants (Supplemental Movie. S1). *DR5:LUC* expression in the root tip increased instantly, irrespective of fluridone treatment, decreasing thereafter (**Fig. 3**). This transient activation of *DR5:LUC* may occur due to the stress caused by the transfer of plants to another medium. Following this artificial elevation, *DR5:LUC* expression remained higher in the fluridone medium compared with that in the control medium after 14 h in WT plants (**Fig. 3A)**; however, the *35S::mHvPDS* plants did not exhibit *DR5:LUC* expression (**Fig. 3B**). These results suggest that PDS inhibition elevated auxin levels and activated auxin signaling.

Auxin biosynthesis and signaling are required for carotenoid-mediated root growth regulation

We hypothesized that fluridone-induced PR growth inhibition is mediated by the elevation of endogenous auxin and activation of auxin signaling. Therefore, mutants of auxin signaling or auxin biosynthesis were examined for fluridone-mediated PR growth inhibition. The roots of the *AUX/IAA* dominant mutants *aux/iaa19/msg2-1* and *aux/iaa14/slr-1* exhibited a moderate but nonsignificant resistance to fluridone. The *ARF* double-mutant *arf7-1 arf19-1* also exhibited a similar level of resistance to that of *aux/iaa19/msg2-1* and *aux/iaa14/slr-1*. An auxin permease mutant *aux1-21* exhibited higher resistance to fluridone compared with the above mutants. A mutant of ubiquitin-activating enzyme E1, *axr1-12* (Lincoln et al., 1990), exhibited the highest resistance to fluridone; however, the resistance of *aux/iaa3/shy2-101* was comparable with that of the WT (**Fig. 4A**), thus indicating that a dominant mutant of the *AUX/IAA* gene does not confer resistance to fluridone.

Auxin biosynthesis and auxin signaling are necessary for PR growth (Mallory et al., 2005; Chen et al., 2014). Anthranilate is a precursor to tryptophane biosynthesis, which is an

intermediate of IAA biosynthesis. Mutants of the anthranilate synthase subunit *wei2* and *wei7* decrease endogenous auxin levels (Stepanova et al., 2005), exhibiting moderate and no resistance to fluridone, respectively, whereas their double-mutant, *wei2 wei7*, was insensitive to fluridone (**Fig. 4B**). *SHADE AVOIDANCE 3* (*SAV3*), which encodes one of five tryptophan aminotransferases in *Arabidopsis*, converts tryptophan to indole-3-pyruvate (Stepanova et al., 2008). A single mutant of *sav3-2* did not demonstrate resistance to fluridone (**Fig. 4B**). *YUCCA* encodes a flavin monooxygenase that catalyzes the last step in auxin biosynthesis, the conversion of IpyA to IAA (Mashiguchi et al., 2011; Won et al., 2011). The quintuple *YUCCA* gene mutants *yucQ* (*yuc 3 5 7 8 9*) decreased the endogenous auxin levels and exhibited severe dwarf shoots and root phenotypes. This dwarf phenotype could be rescued by exposure to exogenous auxin (Chen et al., 2014). Applying fluridone to *yucQ* plants did not inhibit PR growth, instead encouraging it (**Fig. 4C**; >100% of inhibition), suggesting that increased endogenous auxin levels can complement the growth defect in *yucQ* plants. However, a single *YUCCA* gene mutant did not exhibit significant defects in PR growth, such as in the *yucQ* plant, and exhibited less or no resistance to fluridone (Supplemental Fig. S4; Supplemental Table. S1), indicating the functional redundancy of the *YUC* and *SAV3* gene families in this mechanism. These results indicate that carotenoid biosynthesis (i.e., PDS activity) negatively regulates endogenous auxin levels and auxin signaling.

Next, we evaluated the pharmaceutical perturbation of auxin signaling and biosynthesis in combination with PDS inhibition by fluridone. Auxinole targets the auxin binding site of the auxin receptor TIR1/AFB (Hayashi et al., 2012) and inhibits auxin signaling. Yucasin difluorinated analog (YDF) targets the enzymatic activity of YUCCA (Tsugafune et al., 2017). Although auxinole and YDF can alone suppress PR growth, adding fluridone to either inhibitor did not enhance growth inhibition (**Fig. 4, D and E**). These results support the hypothesis that fluridone-mediated PR growth inhibition requires auxin signaling and a certain level of endogenous auxin. The addition of exogenous auxin or an inhibitor of auxin efflux transport, such as N-1-naphthylphthalamic acid (NPA) (Casimiro et al., 2001), also inhibited PR growth in a dose-dependent manner. However, in contrast to auxinole and YDF, fluridone exhibited an additional effect to both exogenous auxin and NPA on PR growth

(Supplemental Fig. S5). These results suggest that fluridone-induced PR growth suppression occurs solely by an elevation of auxin levels and an efflux transporter may not be the major target of PDS inhibition. Taken together, auxin biosynthesis, signaling, and catabolism may be required for carotenoid-mediated PR growth regulation.

Auxin negatively regulates carotenoid content in root

One of the agricultural uses of auxin is as a herbicide, which causes chlorosis in broad-leaf plants (Grossmann, 2010). The auxin-insensitive mutants *arf7/msg1* and *axr1* are resistant to 2,4-dichlorophenoxyacetic acid (2,4-D) and chlorosis (Watahiki and Yamamoto, 1997). Auxin signaling negatively regulates chloroplast development (Kobayashi et al., 2012) and it is reasonable to consider that carotenoid content is also negatively regulated by auxin signaling. To clarify this hypothesis, the level of β-carotene in the root tissue was quantified. Auxinole and YDF increased β-carotene level in the root and fluridone addition reduced β-carotene level regardless of the presence of auxinole or YDF (**Fig. 5, A and B**). The auxin biosynthesis mutants *wei2 wei7* and *yucQ* increased the level of β-carotene more than two-fold compared with that in the WT (**Fig. 5C**). In addition, a negative regulation of carotenoid accumulation by auxin in the shoot tissue was observed (Supplemental Fig. S6). Conversely, the auxin signaling mutants *aux/iaa19/msg2-1* and *axr1-12* exhibited a minor but nonsignificant increase in β-carotene levels compared with that in the WT (**Fig. 5C**). These data suggest that endogenous auxin levels and signaling negatively control β-carotene levels.

Expression of auxin- and carotenoid-related genes

To explore whether carotenoids regulate auxin biosynthesis and signaling at the gene expression level, the expression of auxin-related genes in root tissue was measured following fluridone treatment. The expression of *AUX/IAA19* increased by approximately four-fold at 4 and 8 h following fluridone treatment in the WT but not in the *35S::mHvPDS* plant (**Fig. 6A**). Consistently, the expression of the auxin biosynthesis gene *YUC9* increased by ~2.5 times at 4 and 8 h following fluridone treatment (**Fig. 6B**). The induction time of auxin biosynthesis and signaling gene expression was associated with the induction of endogenous auxin levels (**Fig.**

275 **2**). These data suggest that *PDS* inhibition activates auxin biosynthesis and signaling.

276 Next, we examined the expression of genes downstream of carotenoid biosynthesis or
277 catabolism. *CCD7* and *CCD8* are carotenoid catabolism genes (i.e., apocarotenoid
278 biosynthesis genes) (**Fig. 1A**) (Yuan et al., 2015). *CCD7* and *CCD8* were primarily induced at
279 8 and 24 h in the WT plants, whereas the expression of these genes remained unchanged in
280 the *35S::mHvPDS* plants **(Fig. 6, C and D)**. On the other hand, carotenoid biosynthesis genes
281 *CCR1* and *CCR2*, and catabolism gene *CCD1* were also examined. The expression of these
282 genes were also somewhat induced (Supplemental Fig. S7). These data imply carotenoid
283 catabolism rather than biosynthesis is activated following PDS inhibition.

284

285 Retinal controls auxin content in fluridone-mediated PR growth 286 regulation

287 The target of fluridone was PDS for PR growth inhibition; however, the downstream product
288 of PDS activity responsible for this process remains unknown. The extremely hydrophobic
289 nature of β-carotene made it impossible for the complementation test by exogenous
290 application to the plant, which was inhibited by fluridone (data not shown). Recently, various
291 β-carotene-derived apocarotenoids were reported to be bioactive. Strigolactone is one such
292 apocarotenoids and mutants of strigolactone biosynthesis genes *max3* and *max4* as well as the
293 strigolactone signaling gene *max2* were examined for fluridone-induced PR growth inhibition.
294 However, these mutants exhibited the same response to fluridone as the WT (data not shown)
295 and may not be responsible for fluridone-induced PR growth inhibition. Then, two
296 β-carotene-derived apocarotenoids, β-cyclocitral and retinal, were employed for the
297 complementation test to fluridone. Retinal, but not β-cyclocitral, completely restored
298 fluridone-induced PR growth inhibition (**Fig. 7A**; Supplemental Fig. S8). An auxin-inducible
299 *pIAA19::ELucPEST* reporter (Yamamoto et al., 2017) was used for quantifying auxin
300 signaling. *IAA19* activity increased two-fold in the root tip area following treatment with 10
301 nM 1-naphthaleneacetic acid (NAA), a synthetic auxin. However, this induction was
302 counteracted by cotreatment with retinal (**Fig. 7E**). These data suggest that the reduction of
303 retinal levels enhances auxin levels. If true, retinal may complement the inhibitory effect of

auxin. Indeed, retinal restored NAA-induced PR growth inhibition (**Fig. 7B**). Although retinal did not affect PR growth in the WT, PR growth in the *wei2 wei7* and *yucQ* mutants was significantly decreased (**Fig. 7C**). From these data, it is speculated that retinal negatively regulates endogenous auxin levels; however, no significant reduction in IAA levels was detected in the root of the retinal-treated plants (Supplemental Fig. S9G). Auxin catabolism inhibitor KKI (Fukui et al., 2022) inhibits PR growth in a dose-dependent manner (**Fig. 7D**) like as treatment with exogenous auxin (**Fig. 7B**). Interestingly, retinal did not affect KKI-induced growth inhibition nor did it rescue PR growth inhibition; however, retinal restored exogenous auxin–induced growth inhibition (**Fig. 7, B and D**). Although the retinal levels in fluridone-treated plants remain to be determined, these results indicate that fluridone-induced retinal levels reduction suppresses endogenous auxin catabolism, consequently increasing auxin levels (**Fig. 2**).

Discussion

Herein, we demonstrated the coupling of carotenoid biosynthesis with root growth regulation. We investigated that inhibiting PDS with fluridone suppressed PR growth (**Fig. 1D;** Supplemental Fig. S3). In addition, other PDS inhibitors, such as norflurazon, diflufenican, and picolinafen, suppressed PR growth and increased LR density, comparable with the effects of fluridone (Supplemental Fig. S1). This inhibition of PR growth was caused by PDS activity suppression, as *35S::mHvPDS* plants are less sensitive to fluridone (**Fig. 1D**; Supplemental Fig. S3). PDS activity suppression coincides with elevated endogenous IAA levels and suppressed PR growth (**Fig. 1, D** and **Fig. 2**; Supplemental Fig. S2A). The *msg2-1/aux/iaa19* (Tatematsu et al., 2004) and *slr-1/aux/iaa14* (Fukaki et al., 2002) plants were less sensitive to fluridone compared with the WT (**Fig. 4A**). In fact, *arf7 arf19* (Okushima et al., 2007) exhibited similar resistance to fluridone as that of *msg2-1* and *slr-1* (**Fig. 4A**). The *axr1-12* and *aux1-21* plants exhibited higher resistance to root inhibition by fluridone compared with the other auxin signaling mutants (**Fig. 4A**). The *axr1-12* and *aux1-7* plants were previously reported to exhibit higher resistance to exogenous auxin compared with *slr-1*, *arf7 arf19*, and *msg2-1* (Timpte et al., 1995; Fukaki et al., 2002; Tatematsu et al., 2004; Okushima et al.,

2005), which is relevant to fluridone resistance (**Fig. 4A**). This supports the hypothesis that fluridone-mediated growth inhibition requires auxin signaling and transport. However, no correlation was observed between fluridone resistance and LR formation defects among the mutants, as *msg2-1/aux/iaa19* and *slr-1/aux/iaa14* were highly LR-defective; however, *axr1-12* and *aux1-7* exhibited LR formation defects to a lesser extent. The *shy2-101/aux/iaa3* plant is also an LR-defective mutant (Goh et al., 2012), however, it exhibited sensitivity to fluridone, similar to that of the WT (**Fig. 4A**). *MSG2/AUX/IAA19* and *SLR/AUX/IAA14* expression is regulated by ARF and AUX/IAA during auxin signaling (Tatematsu et al., 2004; Ito et al., 2016), whereas *AUX/IAA3* expression is under the control of cytokinin (Bishopp et al., 2011) and brassinosteroid (Li et al., 2020) signaling. Fluridone activated a higher degree of *DR5:LUC* activity in the root tip in WT, but not in *35S::mHvPDS* (**Fig. 3**). These findings suggest that auxin-mediated gene expression participates in carotenoid-mediated PR regulation. Finally, auxinole, which is an inhibitor of the auxin receptor (Hayashi et al., 2012), inhibited PR growth; however, fluridone addition did not further suppress PR growth (**Fig. 4D**). This result also supports the requirement of auxin signaling in fluridone-mediated PR inhibition. Conversely, the suppression effect of PR growth by NPA and fluridone was additive (Supplemental Fig. S5B). This may exclude the involvement of the auxin efflux pathway in carotenoid-mediated PR growth regulation.

PDS inhibition activity increases auxin levels

Fluridone-induced PDS activity suppression elevates auxin levels in the root tissue, which may exceed the optimal concentration for root growth, while inhibiting root growth in the WT plants (**Fig. 1, D and 2**). In the case of *yucQ* plant, an acceleration of PR growth in *yucQ* plant which has suboptimal levels of auxin and shorter roots compared with those of the WT (Chen et al., 2014), may be explained by fluridone-induced elevation of auxin levels (**Fig. 4C**). In fact, root growth in *yucQ* was enhanced by exogenous auxin (Chen et al., 2014). Single mutants of the *YUC* gene family exhibited less or no resistance to fluridone (Supplemental Fig. S4), suggesting a functional redundancy of the *YUC* gene family in this mechanism. The ectopic expression of *YUC3* in the shoot of *yucQ* plant (*FRO6::XVE::YUC3*)

resulted in elongated hypocotyl (Chen et al., 2014), which is a typical phenotype of auxin overproduction (Boerjan et al., 1995; Delarue et al., 1998). However, the short root of *yucQ* was not rescued by the expression of *FRO6::XVE::YUC3*, suggesting a bare contribution of shoot-derived auxin for root growth (Chen et al., 2014). Therefore, the effect on root growth of fluridone in the WT and *yucQ* is root-specific and not based on shoot-derived auxin. Notably, fluridone-induced elevation of auxin levels was detected in the root but not in the shoot (Supplemental Table 2).

The *wei2-1 wei7-1* plants exhibited a short-root phenotype complemented by exogenous auxin, such as the *yucQ* plant (Stepanova et al., 2005). Conversely, its root growth was not rescued; however, it exhibited total insensitivity to fluridone (**Fig. 4B**). Although the IPA pathway is a major way of auxin biosynthesis, there is a diversification of the auxin biosynthesis pathway after tryptophan (Zhao et al., 2010; Kasahara, 2016). The difference in response to fluridone between the *wei2-1 wei7-1* double-mutant and *yucQ* indicates the role for the alternative pathway of auxin biosynthesis in fluridone-mediated root inhibition. Together with the induction of *IAA19* and *YUC9* following fluridone treatment, (**Fig. 6, A and B**) results all above indicate that fluridone activates auxin signaling and biosynthesis.

The inhibition of auxin signaling and biosynthesis by auxinole and YDF, respectively, inhibited PR growth; however, fluridone addition did not further enhance this suppression (**Fig. 4, D and E**). These results also indicate that auxin biosynthesis and signaling are required to inhibit root growth by fluridone. In contrast to auxinole and YDF, exogenous auxin and fluridone exhibited an additive effect on PR growth suppression (Supplemental Fig. S5A). These pharmaceutical approaches can indicate the optimal level of auxin and auxin signaling required for PR growth as well as whether excessive or insufficient auxin levels can reduce PR growth. Taken together, fluridone-induced PDS inhibition increases endogenous auxin levels, activates auxin signaling, and thus inhibits PR growth.

Interaction between auxin homeostasis and carotenoid catabolism

One of the apocarotenoids, retinal, completely restored PR growth inhibition induced by fluridone (**Fig. 7A**) and NAA (**Fig. 7B**). Retinal is reported to be responsible for periodic root

branching and regulate LR formation (Dickinson et al., 2021). Besides of retinal, apocarotenoid β-cyclocitral is also a regulator for both PR and LR development (Dickinson et al., 2019). Conversely, β-cyclocitral did not rescue fluridone-induced PR growth inhibition (Supplemental Fig. S8). Retinal counteracted the LR induction caused by NAA and tryptophan and mitigated the exaggerated LR formation of the *sur2* mutant (Supplemental Fig. S9, C–E). These findings indicate a negative role of retinal in auxin signaling. Indeed, retinal suppressed the activation of auxin-inducible *AUX/IAA19* promoter by auxin (**Fig. 7E**). Although retinal restored the fluridone-induced PR suppression over the first 2 d (**Fig. 7A**), partial recovery at a higher concentration of retinal occurred on the third day after treatment (Supplemental Fig. S9A). Notably, the rate of PR growth suppression accelerated after 48 h of fluridone treatment (Supplemental Fig. S3). It is hypothesized that the pool of carotenoids (i.e., β-carotene) was highly depleted at this time, and accordingly, the requirement of retinal raised a high level. KKI is an inhibitor of the auxin–amino acid conjugating enzyme GRETCHEN HAGEN 3 (GH3). The KKI-induced auxin catabolism inhibition rapidly elevated endogenous IAA levels, leading to typical phenotype of excessive auxin, including induced LR and adventitious root formation, and reduced PR growth (Fukui et al., 2022). In contrast to the antagonistic effects of exogenous auxin (**Fig. 7B**), retinal did not rescue either of these phenotypes (**Fig. 7E**; Supplemental Fig. S9F), suggesting that retinal may regulate IAA levels through IAA catabolism. Fluridone-activated *CCD7* and *CCD8* expression at 8 and 24 h in the WT plants (**Fig. 6, C and D**). The *CCD7* and *CCD8* genes, which convert 9-*cis*-β-carotene to 9-*cis*-β-apo-10'-carotenal and 9-*cis*-β-apo-10'-carotenal to carlactone, respectively, are induced by auxin (Bainbridge et al., 2005; Hayward et al., 2009). These data suggest that fluridone-induced auxin (**Fig. 2 and 3**) activates the catabolism of β-carotene and suggest the feedback regulation of apocarotenoid biosynthesis. The GENEVESTIGATOR (https://genevestigator.com) (Hruz et al., 2008) database also confirmed that *CCD7* expression is induced between 0.5 and 1 h following exogenous IAA treatment (Supplemental Fig. S10).

Conclusion

Herein, we established a model of carotenoid-mediated auxin regulation and response via the apocarotenoid retinal (**Fig. 8**). The inhibition of carotenoid biosynthesis elevated auxin levels and induced auxin response, which may have reduced retinal levels and promoted endogenous auxin levels and response. We hypothesized that retinal may accelerate IAA catabolism to regulate endogenous auxin levels. Moreover, auxin may regulate carotenoid accumulation through a feedback mechanism involving the expression of genes associated with carotenoid catabolism. This model reveals the hidden part of the carotenoid and auxin interaction to regulate root growth. It also provides insight into the role of the apocarotenoid retinal in root architecture. Finally, the quantitative analysis of retinal and its derivatives as well as their targets, which may regulate auxin homeostasis, is warranted in the future.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis thaliana* mutants and WT plants as well as *PDS* transgenic plants were in the Columbia background, except for *yuc5*, which is in the Landsberg *erecta* background. The *arf7-1 arf19-1*, *sav3-2*, *yuc1*, *yuc2*, *yuc3*, *yuc4-1*, *yuc5*, *yuc6*, *yuc7*, *yuc8*, *yuc9*, *yuc10*, and *yuc11* plants were described previously (Xu et al., 2017). The *axr1-12* (Timpte et al., 1995), *aux1-21* (Roman et al., 1995), *wei2-1*, *wei7-1*, *wei2-1/wei7-1* and *sur2* plants (Stepanova et al., 2005) were obtained from the Arabidopsis Biological Resource Center. The *yucQ* mutant was a generous gift from Dr Hiroyuki Kasahara (Tokyo University of Agriculture and Technology) with an agreement from Dr Yunde Zhao (University of California San Diego). *DR5:LUC* (Moreno-Risueno et al., 2010) was kindly provided from Dr Philip N. Benfey (Duke University). In this study, *DR5:LUC* reporter in the *DR5:LUC/VENUS* line (Toyokura et al., 2019) was observed. The *slr-1* (Fukaki et al., 2002) and *shy2-101* (Goh et al., 2012) mutants were described previously. Fluridone, norflurazon, deflufenican, picolinafen, and retinal were purchased from Fujifilm Wako pure chemical. KKI, YDF, and auxinole were provided by Kei-ichiro Hayashi. Seeds were surface-sterilized using chlorine gas for at least 45 min, suspended in 0.38% agarose, and sown on half-strength Murashige and Skoog (MS) medium

(Duchefa Biochemie) supplemented with 1% (w/v) sucrose, 0.6% (w/v) gellan gum, and 0.5 mM MES (pH 5.8) if not specifically indicated. Stratification was performed at 4°C for 3 d in the dark. Plants were grown on vertically oriented plates at 22°C under constant light if not specifically indicated (80–110 μmol m^{-2} s^{-1}). Stock solutions of phytohormones and inhibitors were prepared in dimethyl sulfoxide and filtered through a 0.45 μM disc filter.

PDS-resistant vector and plant transformation and screening

The plasmid pPDSer1303 was kindly provided by Renée S. Arias (USDA ARS, USA). The *pds* mutation vector construction was described previously (Arias et al., 2006). The plasmids were transformed into *Agrobacterium* strain C58C1 and used to transform *Arabidopsis thaliana* via the dip floral method. *Agrobacterium* harboring the plasmid pCambia1303 without *pds* was used as a control for the transformations and later as a control for herbicide screening. Seeds from T1, T2, and T3 generations were screened with hygromycin (50 mg/L) and fluridone (25 nM) separately. Seedlings that were resistant to hygromycin in T1 were selected in either hygromycin or fluridone (25 nM) screening in T2 and T3. T3 seeds were screened for resistance to fluridone at 25, 50, and 200 nM.

Quantification of LR density and PR growth

Plants were grown on vertical plates for 5 d and then transferred to a new half-strength MS medium with or without inhibitors or phytohormones. Plant images were captured using a flatbed scanner (GT-X980 and DS-50000, EPSON) within 4 d after transfer. The density of emerged LRs from the root–shoot junction beside an adventitious root and the PR growth rate were analyzed with Fiji software (https://fiji.sc/).

Quantification of PR growth via time-lapse imaging

Plants were grown on vertical plates for 4 d and then transferred to a new half-strength MS medium. Plant images were captured using a digital camera (Lumix G5, Panasonic). The interval was 30 min. After 1 d of photography, the seedlings were transferred to a new half-strength MS medium with or without 800 nM fluridone. The PR growth rate was

analyzed using Fiji software (https://fiji.sc/).

Quantification of IAA

Seeds were sown in sterilized 300 μm nylon mesh and transferred to control or fluridone plates after a 5-d germination together with mesh. Shoot and root samples were harvested at specific timepoints after transfer and frozen in liquid nitrogen. Hormonome analysis was conducted as previously described (Xu et al., 2017). Briefly, samples of ~100 mg fresh weight (Fw) were suspended in 80% (v/v) aqueous methanol with [$^2H_5$]-IAA as internal standards. The samples were homogenized and the supernatant was loaded onto a Bond Elut C18 cartridge (100 mg, 3 ml; Agilent Technologies) and eluted with 80% (v/v) aqueous methanol. The concentrated samples were subjected to liquid chromatography with electrospray ionization tandem mass spectrometry comprising a quadrupole tandem mass spectrometer (Agilent 6460 Triple Quadrupole mass spectrometer) with an electrospray ion source and an Agilent 1200 separation module. The raw data were extracted from MassHunter software (Agilent Technologies) and examined in Excel (Microsoft).

Bioluminescence imaging analysis

D-Luciferin was purchased from Gold Biotechnology. For *DR5:LUC* expression, seeds were germinated on medium containing 100 μM D-luciferin. Five-day-old plants were transferred to 100 μM D-luciferin medium with or without fluridone, following which time-lapse imaging was performed using a camera (ASI294MMPro, ZWO) with control software (SharpCap, v4.0). For the retinal treatment of *pIAA19::ELucPEST*, seeds were germinated on 100 μM D-luciferin medium. Four-day-old plants were transferred to 100 μM D-luciferin medium for preincubation. After 1 d, seedlings were transferred to 100 μM D-luciferin medium with NAA or NAA and retinal and time-lapse imaging was continued. The images were captured at an interval of 1 h, including 15 min of exposure and 45 min of red and blue light (100 μmol m^{-2} s^{-1}) for 5 d and then saved in a TIFF format for further analysis using Fiji software (https://fiji.sc/).

507 **RNA isolation and quantitative reverse transcription polymerase chain reaction**
508 **analysis**

509 Seeds were sown on control medium with 300 μm nylon mesh. Roots were placed on plates
510 containing no herbicide (mock) or 800 nM fluridone, root samples were harvested at 0, 4, 8,
511 and 24 h and frozen in liquid nitrogen. Total RNA was extracted and purified using an RNA
512 isolation kit (ISOSPIN Plant RNA, NIPPON GENE). Complementary DNA was synthesized
513 from the total RNA according to the manufacturer's instructions (ReverTra Ace qPCR RT
514 Master Mix, Toyobo). Quantitative reverse transcription polymerase chain reaction (qRT–
515 PCR) was performed in optical 96-well plates via the LightCycler 480 II system (Roche Life
516 Science) using KOD SYBR qPCR Mix (Toyobo). Primer pairs spanning exon–exon junctions
517 were designed using the QuantPrime program to avoid genomic DNA amplification as listed
518 in Supplemental Table S3 (Xu et al., 2017). The specificity of the reactions was verified via
519 melt curve analysis and capillary electrophoresis (Multina, Shimadzu). Standard curve
520 analysis was used to evaluate the efficiency of the reactions. ACTIN2 was used as an internal
521 standard. The qRT–PCR program included one cycle at 98°C for 2 min, followed by 40 cycles
522 of 98°C for 10 s, 60°C for 10 s, and 68°C for 30 s. The cycle time values were determined
523 using the second derivative maximum method (Tichopad et al., 2003) via LightCycler
524 software (version 1.5, Roche Life Science). The data were analyzed using the comparative
525 threshold cycle (Ct) method ($2^{-\triangle\triangle Ct}$) (Schmittgen and Livak, 2008).
526

527 **Quantification of carotenoid via high-performance liquid chromatography**
528 **analysis**

529 Seeds were sown on control medium with 300 μm nylon mesh. The seedlings were grown
530 under the conditions described above. Five days after germination, the seedlings together with
531 nylon mesh were transferred to plates with or without fluridone and with or without NAA or
532 auxinole or YDF. Shoot tissues (~20 mg) or root tissues (~100 mg) were harvested at specific
533 timepoints and stored at −80°C. Total carotenoids were extracted using acetone wako 1st grade
534 (FUJIFILM Wako Chemicals). Acetone was added to the tissue samples that had been mixed
535 with beads and silica gel and shaken for 2 min. Total carotenoids were separated via

536 centrifugation for 10 min at 14000 rpm at 4℃. High-performance liquid chromatography
537 analysis for carotenoid quantification was performed as previously described (Zapata et al.,
538 2000). An aliquot of the sample was subjected to a C8 column (4.6 × 150 mm, Waters
539 Symmetry C8, Waters) equilibrated with A solution (methanol:acetonitrile:aqueous pyridine
540 solution [0.25 M pyridine] = 2:1:1 [v:v:v]). Pigments were eluted with a linear gradient of
541 0%–40% B solution (methanol:acetonitrile:acetone = 1:3:1 [v:v:v]) for 2 min, 40%–95% for 6
542 min, an isocratic elution for 5 min, a linear gradient of 95%–0% for 2 min, and an isocratic
543 elution for 7 min at a flow rate of 1 mL/min. Pigments were identified based on their retention
544 time relative to known standards and quantified by peak area on the chromatogram.
545

546 ## Statistical analysis

547 In this study the statistical significance was determined by 2-tailed Student's *t*-test (*: $P <$
548 0.05, **: $P < 0.01$) or one-way ANOVA following Tukey-Kramer test (Different lowercase
549 letters indicate significant differences, $P < 0.05$).
550

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555

556 ## Author contributions

557 Author contributions: K.X. conceived the project and designed the experiments, which were
558 mostly carried out and analyzed by K.X.; H.Z. supported retinal-related experiment design,
559 process and data analysis; E.Y. and M.A. were responsible for IAA quantification and data
560 collection; K.X. and M.K.W. wrote the article. K.X., H.Z., E.Y., M.A., K.H., H.F., H.I. and
561 M.K.W. read, commented and approved the final manuscript.
562

563 ## Supplemental data

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

Supplemental Figure S1. Other PDS-target inhibitors exhibit a fluridone-like effect on PR suppression and LR induction.

Supplemental Figure S2. Long-period auxin level change by fluridone.

Supplemental Figure S3. Kinetic PR growth suppression of WT and *mPDS* plants by fluridone.

Supplemental Figure S4. *yuc* single mutants have weak or no resistance to fluridone.

Supplemental Figure S5. Exogenous auxin and NPA suppress PR in an additive manner with fluridone.

Supplemental Figure S6. Carotenoid in shoot tissue is negatively regulated by auxin.

Supplemental Figure S7. Relative expression level change of carotenoid biosynthesis genes (*CCR1*, *CCR2*) and the metabolism gene *CCD1*.

Supplemental Figure S8. β-cyclocitral is not involved in carotenoid-mediated PR regulation.

Supplemental Figure S9. Retinal is responsible for fluridone effect and regulates downstream auxin content and response.

Supplemental Figure S10. *CCD7* expression is rapidly induced by exogenous IAA treatment.

Supplemental Table S1. PR growth rate in no fluridone-treated auxin-related mutants.

Supplemental Table S2. Endogenous IAA concentration in fluridone treatment in shoot tissue.

Supplemental Table S3. Gene-specific primers used for qRT–PCR analysis.

Supplemental Movie S1. *DR5:LUC* bioluminescence video under fluridone treatment in WT and *mPDS*.

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Figure legends

Figure 1. Inhibition of PR growth by fluridone mediates phytoene desaturase activity. A) Carotenoid biosynthesis pathway. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CCR1/SDG8, carotenoid chloroplast regulatory1/set domain group 8; CCR2/CRTISO, carotenoid chloroplast regulatory2/crtiso; β-LCY, β-cyclase; CCD1/4/7/8, carotenoid cleavage dioxygenase 1/4/7/8. **B)** Five-day-old plants were transferred to medium with or without 12.8 μM fluridone. Photographs were captured 3 d after transfer. Scale bar = 5 mm. **C)** Leaf pigment bleaching effect of WT (left) and *mPDS* (right) by fluridone. Five-day-old plants were transferred to medium with or without 800 nM fluridone. Photographs were captured 3 d after transfer. Scale bar = 1 mm. **D**–**F)** Five-day-old plants were transferred to medium with or without fluridone. PR growth rate **(D)** was analyzed from 2 d after transfer (dat) to 3 dat. LR density **(E)** was counted at 3 dat. Total carotenoid (lutein, neoxanthin, violaxanthin, and β-carotene) **(F)** was quantified at 2 dat. Data represent the means ± standard error (SE) from 12 seedlings in **(D**, **E)** and from 3 biological replicates in **(F)**. *Significant differences compared to WT in **(D**–**F)** (*: $P < 0.05$, **: $P < 0.01$; Student's *t*-test).

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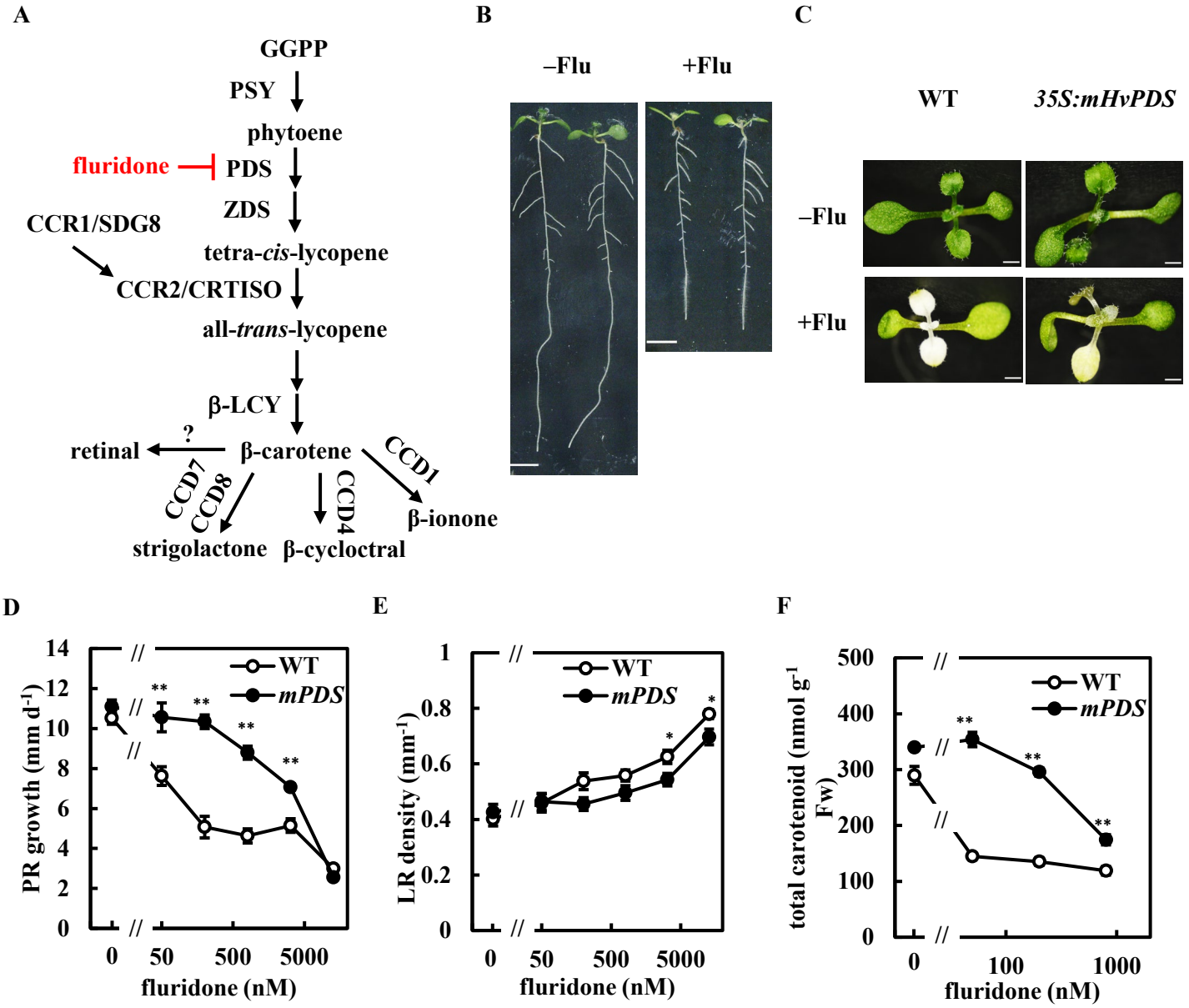

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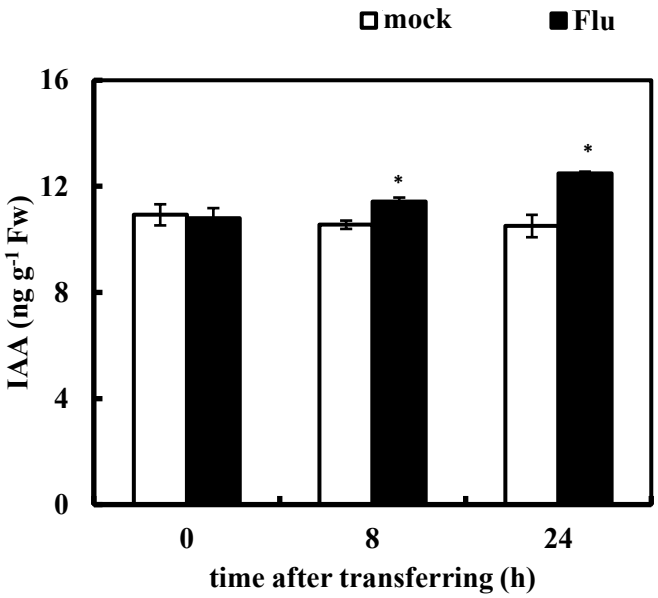

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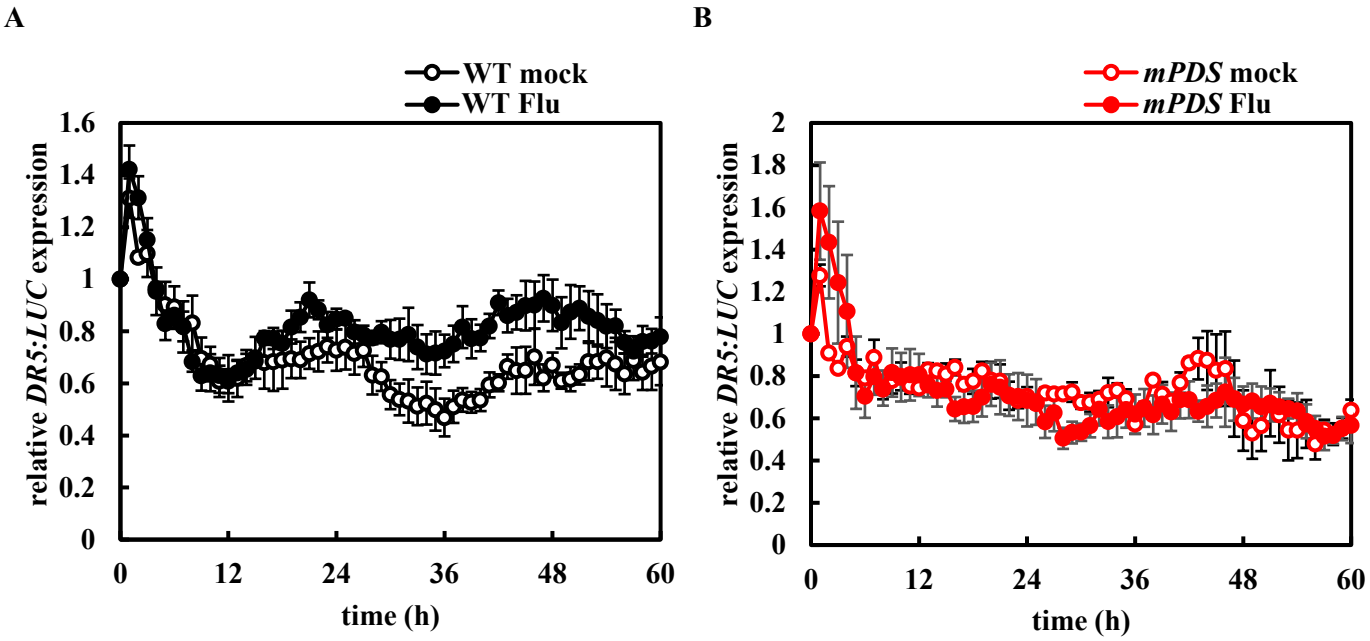

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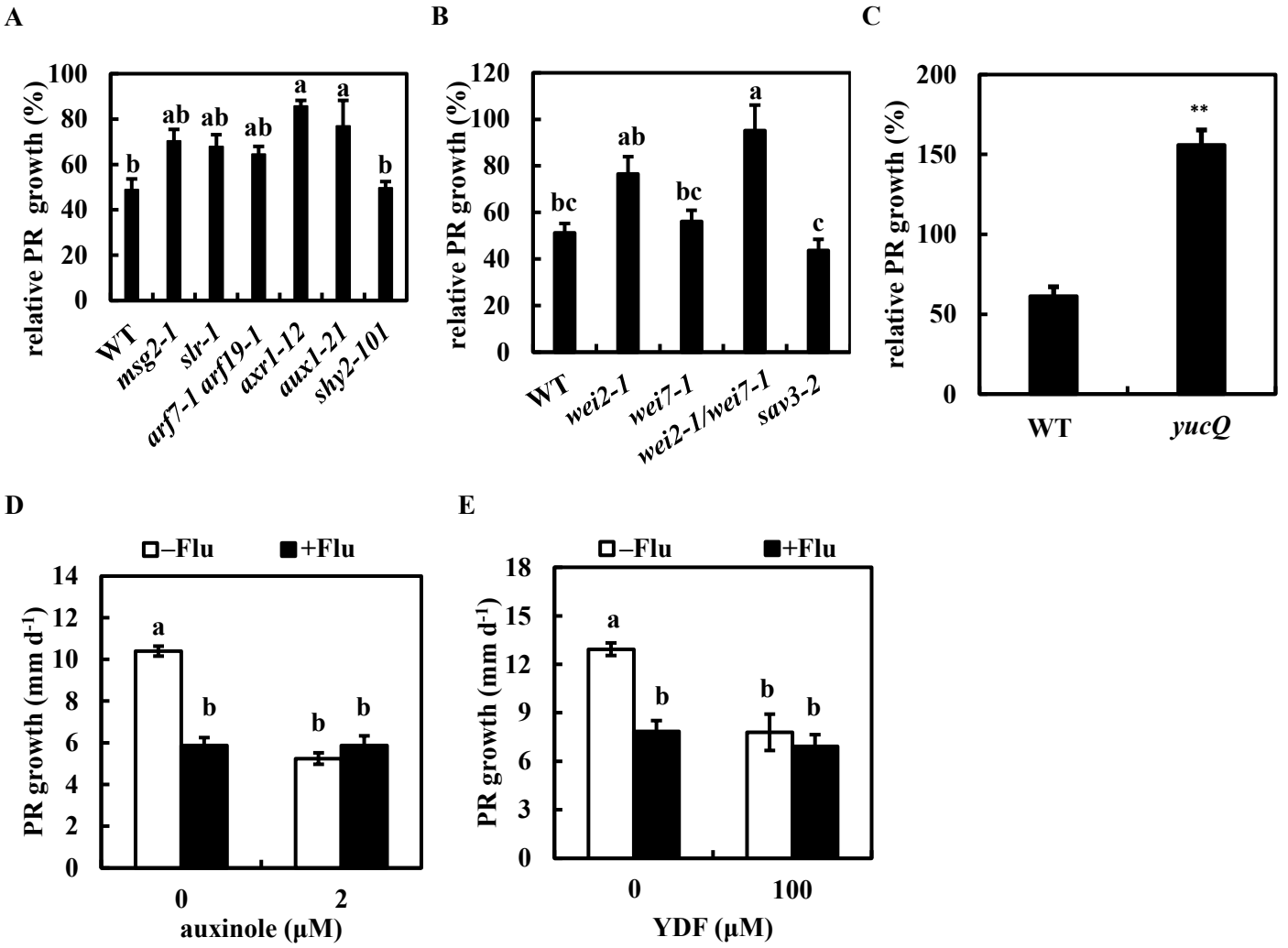

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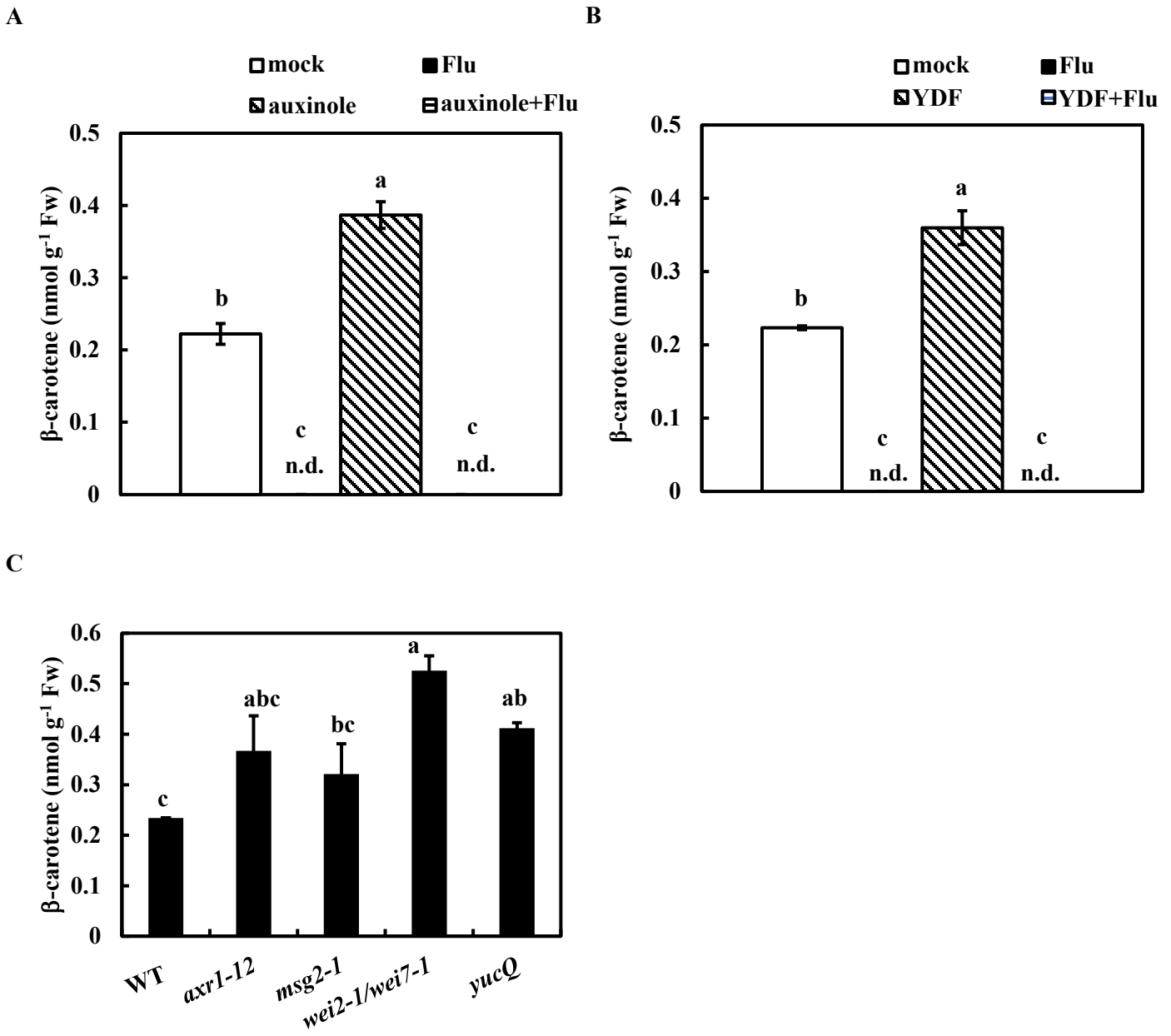

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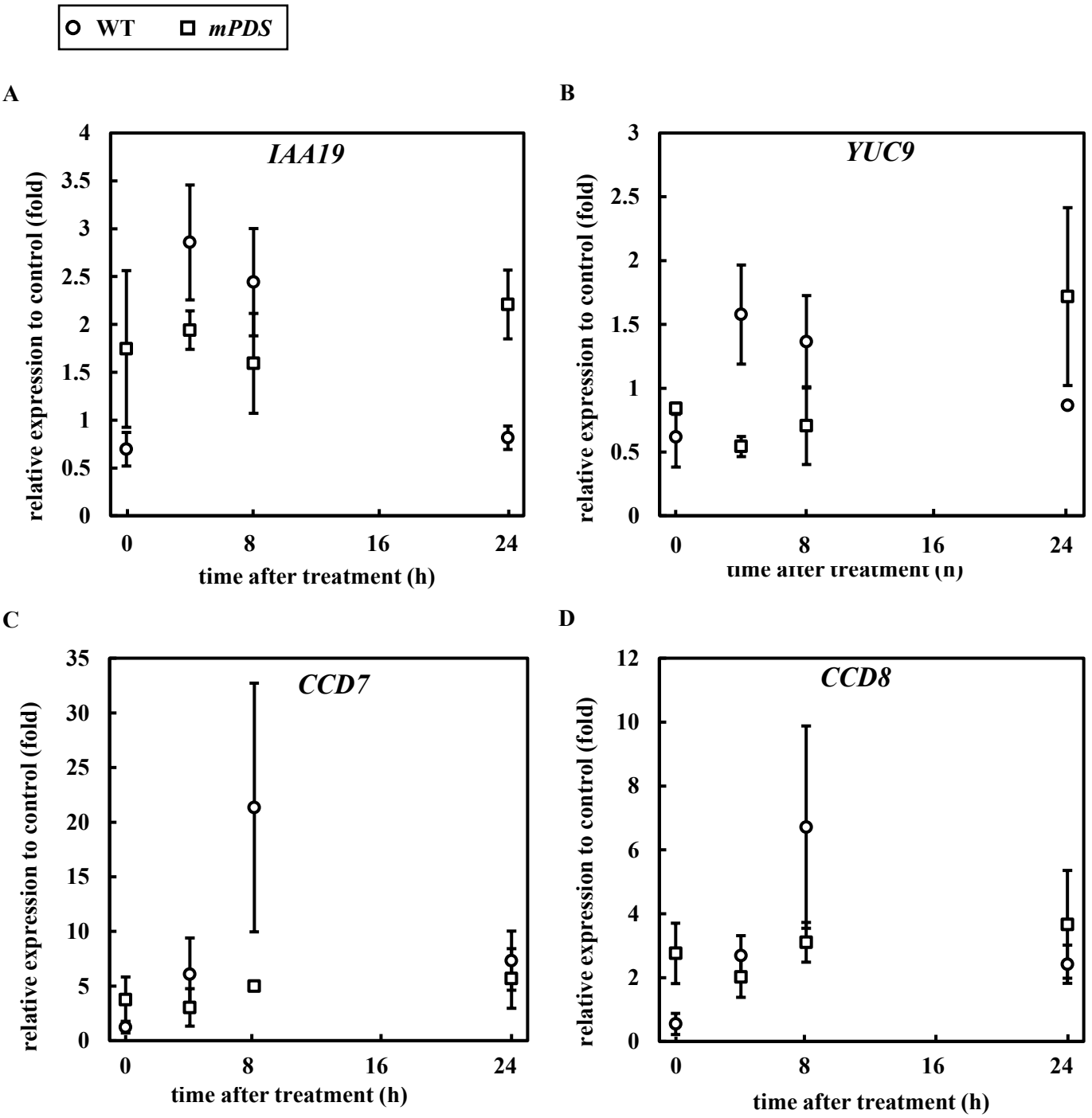

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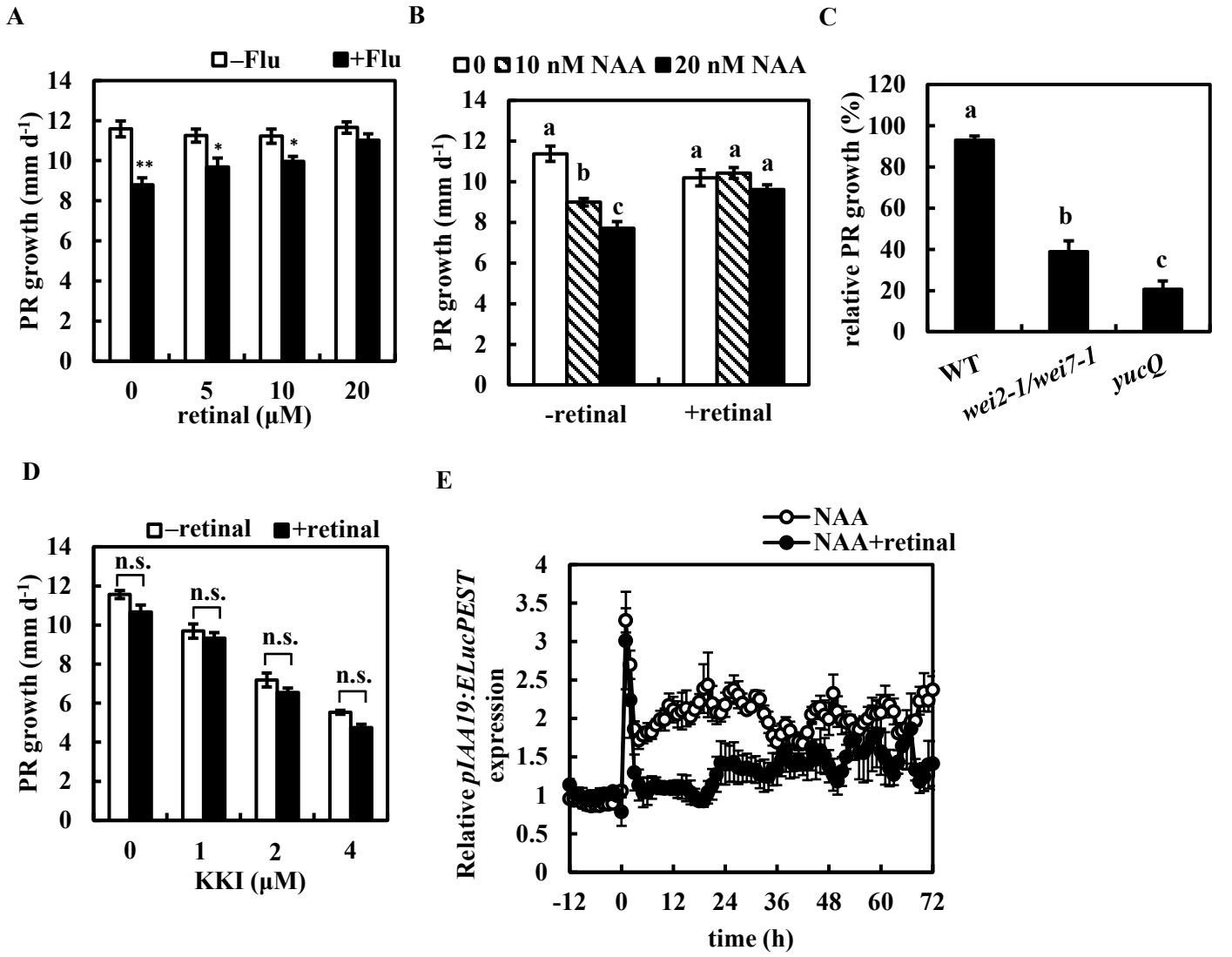

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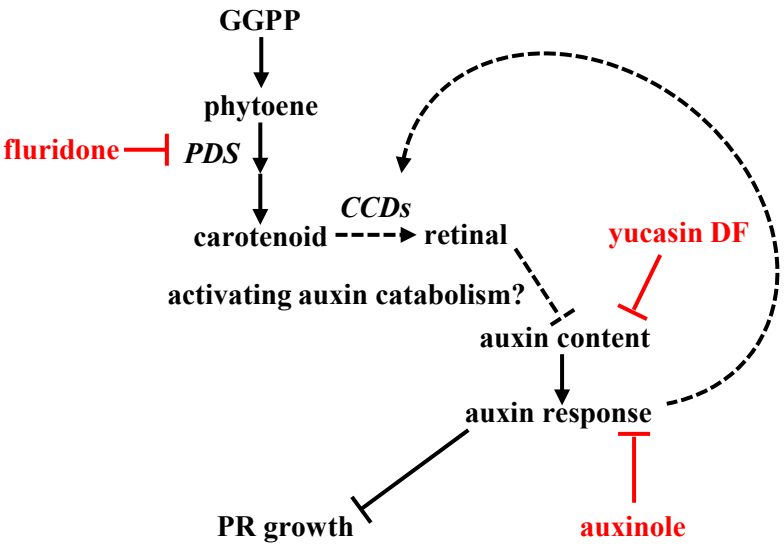

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