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Molecular genetic study of carotenoid-mediated root development
(カロテノイドを介した根の発達の分子遺伝学的研究)

A DISSERTATION
submitted to the Graduate School of Life Science,
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DOCTOR OF LIFE SCIENCE

By
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Kang Xu, Masaaki Watahiki: Identification of specific inhibitor for root-cutting response in *Arabidopsis thaliana* and involvement of phytohormone response. The 61st Annual Meeting of the Japanese Society of Plant Physiologists (令和 2 年, 大阪, 日本)

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5. 特許

なし

Abstract

The plant root system plays a pivotal role in supporting plant development in various aspects, including anchoring, water and nutrient absorption, and signaling in root–shoot interactions. The root system architecture (RSA) comprises primary root (PR) and secondarily developed lateral roots (LRs). The elongation and branching of roots, as one of the main strategies for plant survival, are under the control of metabolism of endogenous metabolites (e.g., carotenoids, apocarotenoids). Under *in vitro* conditions with sucrose application, carotenoid deficiency does not lead to plant death but reduces root elongation. The phytohormones abscisic acid (ABA) and strigolactones (SLs) which are derived from carotenoids, have fundamental roles in root growth regulation (Zhang et al., 2010; Ruyter-Spira et al., 2011; Thole et al., 2014). In recent years, the discovery of bioactive apocarotenoids has increased (Dickinson et al., 2019; Jia et al., 2019; Dickinson et al., 2021). Among them, retinaldehyde (retinal), a precursor of vitamin A required for vision in animals, is identified as a regulator in periodic LR patterning (Dickinson et al., 2021). Although the metabolism pathway and roles of retinal in animals are well documented (Lidén and Eriksson, 2006; Raghuvanshi et al., 2015), the roles of this compound in plants are still largely unknown. On the other hand, carotenoids and apocarotenoids have been reported to be involved in reactive oxygen species (ROS) and respond to various stress (e.g., high light, drought and salt tolerance) (Ramel et al., 2012; D'Alessandro et al., 2019). Plants also show capable ability of organ regeneration for the adaptation to the environmental conditions (e.g., physical damage). The injury of PR encourages the elongation and branching of LRs, which is broadly applied in agricultural and horticultural fields as a technique called “root pruning”. The induction of LR branching by physical damage of PR has been partially clarified (Xu et al., 2017); however, the acceleration of its growth remains elusive, and the involvement of apocarotenoids in this regulation is unclear. In this study, I aim to determine the roles of apocarotenoids in the regulation of PR growth, and the LR growth induced by PR damage.

In the first chapter of this thesis, the regulation mechanism of auxin-mediated root growth regulation via the apocarotenoid retinal was investigated. I found 1) Short-term treatment of carotenoid biosynthesis inhibitor fluridone suppressed PR growth and induced LR density. 2) Fluridone-mediated suppression of PR growth is dependent on phytoene desaturase (PDS) activity. 3) Fluridone treatment elevated endogenous auxin levels and activated auxin signaling in roots. 4) Retinal restored fluridone-induced PR growth suppression and negatively regulated auxin levels possibly via auxin catabolism. These results suggest a regulation mechanism of PR growth by retinal, in which retinal may positively control auxin catabolism to reduce auxin levels and response.

In the second chapter of this thesis, I partially revealed the mechanism of root-cut-induced LR growth (RCG) regulation. In this study, I found 1) Fluridone suppressed RCG possibly via the auxin pathway. 2) β -cyclocitral and β -ionone are responsible for fluridone-mediated RCG regulation. 3) Root-cut reduced endogenous cytokinin levels. 4) Cytokinins negatively regulate RCG. 5) Chromatin remodeling factor PICKLE is necessary for RCG possibly via the cytokinin signaling pathway. These results indicate that the carotenoid and apocarotenoid biosynthesis pathways positively contribute to RCG. Besides, cytokinins may be a key regulator in RCG, though the interaction between the cytokinin and carotenoid pathways is yet to be revealed in the future.

The apocarotenoids have a large diversity of functions in plants and animals. In animals, the precursor to vitamin A, retinal, is essential for visual phototransduction, which is the initial step in the light-detection process of vision. (Wong, 1999; Lidén and Eriksson, 2006). However, the functions of this important apocarotenoid in plants, especially in root development, have been recently documented (Dickinson et al., 2021), indicating that there is still much to explore in this research field. Besides, there are other bioactive apocarotenoids reported to participate in RSA regulation (e.g., β -cyclocitral, anchorene) (Dickinson et al., 2019; Jia et al., 2019), suggesting a deep interaction between apocarotenoids and root development. In this study, I investigated that the carotenoids and apocarotenoids are important regulators for PR growth and root-cut-induced LR growth. I showed that carotenoid metabolism negatively controls

244 endogenous auxin levels via apocarotenoid retinal to regulate PR growth. I observed
245 apocarotenoids are responsible for fluridone-mediated LR growth induced by physical
246 damage. I also revealed that the LR growth induced by physical damage is negatively
247 regulated by the phytohormone cytokinins. This study will help elucidate the roles of
248 apocarotenoids in regulating RSA development, which is distinct from their roles in
249 animals.

250 **Abbreviations**

251	2,4-D, 2,4-dichlorophenoxyacetic acid
252	AAO3, ABSCISIC ALDEHYDE OXIDASE 3
253	ABA, abscisic acid
254	ABA1, ABA DEFICIENT 1
255	ABCB/MDR/PGP, ATP-binding cassette B /MULTIPLE DRUG RESISTANCE/P-
256	GLYCOPROTEIN
257	<i>abi</i> , <i>aba</i> insensitive
258	AFB, AUXIN SIGNALING F-BOX PROTEIN
259	AHK, <i>Arabidopsis</i> histidine kinase
260	AHP, <i>Arabidopsis</i> histidine phosphotransferase protein
261	AM, arbuscular mycorrhizal
262	ARF, AUXIN RESPONSE FACTOR
263	ARR, <i>Arabidopsis</i> response regulator
264	Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID
265	AXR1, AUXIN RESISTANT 1
266	BCO1, β -carotene oxygenase 1
267	CCD, CAROTENOID CLEAVAGE DIOXYGENASE
268	CCR2/CRTISO, CAROTENOID CHLOROPLAST REGULATOR2/CAROTENOID
269	ISOMERASE
270	CHD, chromodomain-Helicase-DNA
271	CKH2, CYTOKININ HYPERSENSITIVE2
272	CKX, CYTOKININ OXIDASE
273	Ct, comparative threshold cycle
274	cZ, <i>cis</i> -zeatin
275	D27, DWARF27
276	dac, day after root-cut
277	dat, day after transfer

278 DMAPP, dimethylallyl diphosphate
 279 *DR5:LUC, DR5:Luciferase*
 280 DZ, dihydrozeatin
 281 Fw, fresh weight
 282 GGPP, geranylgeranyl pyrophosphate
 283 H3K27me3, H3 lysine 27 trimethylation H3K27me3
 284 HAT, histone acetyltransferase
 285 IAA, indole-3-acetic acid
 286 IAM, indole-3-acetamide
 287 IAN, indole-3-acetonitrile
 288 IAOx, indole-3-acetaldoxime
 289 IG, indole-glucosinolate
 290 iP, N⁶- (Δ^2 -isopentenyl) adenine
 291 IPT, adenosine phosphate-isopentenyltransferase
 292 IPyA, indole-3-pyruvic acid
 293 KKI, kakeimide
 294 LAX, LIKE AUX1
 295 LBO, LATERAL BRANCHING OXIDOREDUCTASE
 296 LR, lateral root
 297 MAX, MORE AXILLARY GROWTH
 298 MeCLA, methyl carlactonoate
 299 MEP/DOXP, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate
 300 MS, Murashige and Skoog
 301 *msg2/aux/iaa19, massugu2/auxin/indole-3-acetic acid19*
 302 MVA, mevalonate
 303 NAA, 1-naphthaleneacetic acid
 304 NCED, 9-*cis* epoxycarotenoid dioxygenase
 305 NPA, N-1-naphthylphthalamic acid
 306 *pde226, pigment defective 226*

307 PDS, PHYTOENE DESATURASE
 308 PKL, PICKLE
 309 PKR, PICKLE RELATED
 310 PIN, PINFORMED
 311 PP2C, type 2C protein phosphatases
 312 PSY, PHYTOENE SYNTHASE
 313 PR, primary root
 314 PYR/PYL/RCAR, PYRABACTIN RESISTANT/PYRABACTIN RESISTANT-
 315 LIKE/REGULATORY COMPONENT OF ABA RESPONSE
 316 *pyrQ, pyr1pyl1pyl2pyl4*
 317 QC, quiescent center
 318 qRT–PCR, quantitative reverse transcription polymerase chain reaction
 319 RCG, root-cut induced LR growth
 320 RCN, root-cut induced LR number
 321 retinal, retinaldehyde
 322 ROS, reactive oxygen species
 323 RSA, root system architecture
 324 RSJ, root–shoot junction
 325 SDG8/CCR1, SET DOMAIN GROUP 8/ CAROTENOID CHLOROPLAST
 326 REGULATOR1
 327 *shy2/aux/iaa3, suppressor of hy2 mutation 2/auxin/indole-3-acetic acid3*
 328 SL, strigolactone
 329 *slr/aux/iaa14, solitary root/auxin/indole-3-acetic acid14*
 330 SnRK2, snf1-related protein kinases 2
 331 SSR2, SUPPRESSOR OF SLR2
 332 *SUR2, SUPERROOT2/CYP83B1*
 333 TAA, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS
 334 TIR1, TRANSPORT INHIBITOR RESPONSE 1
 335 tZ, *trans*-zeatin

336 WEI2/ASA1, WEAK ETHYLENE INSENSITIVE 2/ANTHRANILATE SYNTHASE
 337 α 1
 338 WEI7/ASB1, WEAK ETHYLENE INSENSITIVE 7/ANTHRANILATE SYNTHASE
 339 β 1
 340 WT, wild-type
 341 YDF, yucasin difluorinated analog
 342 YUC, YUCCA
 343 *yucQ*, *yucca* 3 5 7 8 9
 344 ZDS, ZETA-CAROTENE DESATURASE

General introduction

The root system plays a vital role in water and nutrient uptake, anchoring plants to the soil, and serving as a source of signaling chemicals. *Arabidopsis thaliana* (Arabidopsis) belongs to the eudicot family and has a taproot system which consists of an embryonic developed primary root (PR) and postembryonic developed lateral roots (LRs). The root system architecture (RSA), which includes the elongation of PR and the branching of LRs, is controlled by environmental cues (e.g., physical damage) and endogenous metabolites (e.g., carotenoids and apocarotenoids). The development of RSA enables plants to adapt to the environment.

Carotenoids are a diverse group of plastidial isoprenoid pigments essential for photosynthesis (Ruiz-Sola and Rodríguez-Concepción, 2012). They provide plants with distinct colors ranging from yellow to red. In the absence of carotenoid biosynthesis, plants display leaf albinism and lose the ability of photosynthesis. Besides their functions as photopigments, they also play roles in the regulation of RSA. *phytoene desaturase 3* (*pds3*) and *phytoene synthase* (*psy*) mutant which lacks carotenoid biosynthesis have short PRs and few or no LRs. There are a series of herbicides targeting PDS to inhibit carotenoid biosynthesis and bleach leaf pigments (e.g., fluridone and norflurazon). The application of these inhibitors also suppresses PR growth, by which the mechanisms remain elucidated.

On the other hand, RSA is also affected by abiotic stress such as physical damage. An agricultural technique known as root pruning, which involves the removal of part of the roots, is widely used to improve the quality of fruits (Schupp and Ferree, 1987). This technique is also employed to regulate plant size and maintain vigor, as is the case in the production of bonsai plants. The induction of LR formation by removing the root tips has long been documented, and part of the mechanism has been clarified before by using a mimic technique of root pruning, called root cutting (Xu et al., 2017). However, the regulation of LR elongation induced by root cutting remains to be elucidated.

In this study, I examined the underlying mechanisms of PR growth regulated by

carotenoids and apocarotenoids, particularly the short-term inhibition of PDS activity. I also investigated the participation of carotenoids and apocarotenoids in regulating LR growth induced by root cutting.

Auxin is a key regulator of root development

The phytohormone auxin is known to affect almost every aspect of root development. In *Arabidopsis*, auxin is demonstrated to play vital roles in PR elongation, LR initiation, root gravitropism, and root hair formation (Benjamins and Scheres, 2008; Fukaki and Tasaka, 2009; Moreno-Risueno et al., 2010; Petricka et al., 2012; Saini et al., 2013; Chen et al., 2014). The application of exogenous auxin suppresses PR length and induces LR formation (Hobbie and Estelle 1994; Okumura et al., 2013). Auxin signaling occurs when auxin acts as molecule glue, bringing AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins together with the E3 ligase complex TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN (SCF^{TIR1/AFB}). The SCF^{TIR1/AFB} complex then recognizes and ubiquitinates Aux/IAA proteins, leading to their degradation. This inactivates the functional repression of the transcription factor AUXIN RESPONSE FACTOR (ARFs) by Aux/IAA, thus activating auxin signaling (Leyser, 2002; Shimizu-Mitao and Kakimoto, 2014; Leyser, 2018). Gain-of-function mutations of typical *aux/iaa* exhibit insensitivity to exogenous auxin addition and are accompanied by LR defect (i.e., *massugu2/auxin/indole-3-acetic acid19* [*msg2/aux/iaa19*] [Tatematsu et al., 2004], *solitary root/auxin/indole-3-acetic acid14* [*slr/aux/iaa14*] [Fukaki et al., 2002], and *suppressor of hy2 mutation 2/auxin/indole-3-acetic acid3* [*shy2/aux/iaa3*] [Goh et al., 2012]). Overexpression of *ARF19* reduces PR length, suggesting a negative role for auxin signaling in PR elongation (Okushima et al., 2005). The auxin influx carriers 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 RSA (Vieten et al., 2007).

The auxin permease mutant *aux1* exhibits both agravitropism and resistance to PR growth suppression by exogenous IAA (Yamamoto and Yamamoto, 1998). The longer PRs of *aux1* compared to wild-type (WT) also suggest a negative role of endogenous auxin in PR growth (Roman et al., 1995).

Auxin biosynthesis pathway

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 prevents herbivores and 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* and a allele of *sur2* mutant, *fewer roots suppressor1* (*fsp1*), accumulates IAA for an overaccumulation of tryptophan 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 α 1 (WEI2/ASA1), and WEI7/ANTHRANILATE SYNTHASE β 1 (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).

Carotenoid biosynthesis pathway

Carotenoids are a group of plastidial isoprenoid pigments that are 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 PSY, which undergoes desaturation by 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. 1.1). Carotenoids exhibit diverse functions. They act as protective photopigments in the photosynthetic complex and membranes, add color to fruit, and are a source of bioactive apocarotenoids, which are the products of oxidative cleavage of carotenoids (Cazzonelli, 2011). The apocarotenoid phytohormone abscisic acid (ABA) as well as strigolactones (SLs) are well characterized (Schwartz et al., 1997; Umehara et al., 2008), there has been an increase in the number of new bioactive apocarotenoids discovered during this century (Auldridge et al., 2006; Zheng et al., 2021). β -cyclocitral is volatile and has been found in *Arabidopsis thaliana*, *Solanum lycopersicum* (tomato), *Citrus*, *Vitis vinifera* (grape), *Citrullus lanatus* (watermelon) and *Crocus sativus* (saffron) (Havaux, 2020). It can be derived from β -carotene by the cleavage of C7, C8 position (Rodrigo et al., 2013). Recently, it has been identified as a root growth regulator and exhibits a positive effect on PR elongation and LR capacity (Dickinson et al., 2019). Anchorene is a newly identified carotenoid-derived dialdehyde (diapocarotenoid) and formed by cutting the C11, C12 and C11', C12' bonds in almost all plant carotenoids. It promotes adventitious root formation by modulating auxin

distribution (Jia et al., 2019). Another example is retinaldehyde (retinal), a precursor of vitamin A and retinoic acid in vertebrates (Olson and Hayaishi, 1965). Retinal is found to be formed by the cleavage at C15, C15' position of β -carotene by β -carotene oxygenase 1 (BCO1) in animals (Raghuvanshi et al., 2015). Although the existence of retinal is confirmed (Koschmieder et al., 2021), its biosynthesis is still a mystery in flowering plants. Recently, it has been found to play a role in the periodic branching of the root (Dickinson et al., 2021). In addition to these bioactive apocarotenoids, ionone derivatives such as α - and β -ionone which are often found in the scent of flowers such as *Rosa hybrida* (hybrid tea rose), *Osmanthus fragrans* (sweet osmanthus), *Brassica napus* and *Freesia hybrida*, as well as in the aroma of fruits and vegetables such as *Averrhoa carambola* (starfruit) and or *Lycopersicon esculentum* (tomato). The primary function of ionone derivatives is to repel or attract plant herbivores (Dudareva et al., 2006). Bixin, mainly found in *Bixa orellana* seeds, and safranal, primarily found in saffron, are also important in the food industry as color additives (Zheng et al., 2021).

The inhibitors of the carotenoid biosynthesis pathway

Fluridone and norflurazon are the herbicides used for eliminating aquatic plants and inhibiting PDS activity, resulting in chlorophyll degradation and chloroplast membrane destruction, which is characterized by a tissue bleaching phenotype and the suppression of carotenoid-derived phytohormones (e.g., ABA and SLs) (Bartels and Watson, 1978; Yoshioka et al., 1998; López-Ráez et al., 2008). Arabidopsis seedlings grown on media containing these kinds of PDS inhibitors show a lack of carotenoids, accumulation of phytoene, and reduced integrity of chloroplasts (Kim and Apel, 2013). The RSA, including PR elongation and LR formation, is also severely arrested by carotenoid biosynthesis inhibitors (Van Norman et al., 2014).

Phytohormone ABA affects root development via the interaction with other phytohormones

ABA was discovered in the early 1960s, for its role in regulating senescence and stress

responses in the abscising organs. ABA has been found among almost all clades of organisms, including cyanobacteria, algae, lichens, sponges, plants, and mammals. ABA biosynthesis begins with isoprenoids derived from the MEP pathway. The zeaxanthin epoxidase ABA DEFICIENT 1 (ZEP/ABA1) converts zeaxanthin into violaxanthin. Zeaxanthin and violaxanthin can be converted into 9'-*cis*-neoxanthin and 9'-*cis*-violaxanthin, respectively. The rate-limiting step in the ABA biosynthesis pathway is usually thought to be the conversion of 9'-*cis*-neoxanthin and 9'-*cis*-violaxanthin into xanthoxin through the 9-*cis* epoxycarotenoid dioxygenase (NCED) family of enzymes. Xanthoxin is then converted to abscisic aldehyde by ABA2. Finally, abscisic aldehyde is catalyzed by ABSCISIC ALDEHYDE OXIDASE 3 (AAO3) which is activated by a molybdenum cofactor ABA3, to produce ABA (Nambara and Marion-Poll, 2005; Finkelstein, 2013). ABA signaling is started with the perception by the proteins of PYRABACTIN RESISTANT/PYRABACTIN RESISTANT-LIKE/REGULATORY COMPONENT OF ABA RESPONSE (PYR/PYL/RCAR) family, the receptors of ABA. Upon ABA binding, PYR/PYL/RCARs interact with type 2C protein phosphatases (PP2Cs), releasing snf1-related protein kinases 2 (SnRK2) from PP2C-dependent repression and activating downstream ABA response genes (Ma et al., 2009; Park et al., 2009; Cutler et al., 2010). Many studies have gone into my understanding of ABA about its functions on root architecture regulations and interactions with other phytohormones. While ABA is commonly perceived as a negative regulator of PR and LR growth (De Smet et al., 2003; Thole et al., 2014), it is also reported to be essential for quiescent center (QC) cell maintenance and meristem cell differentiation and consequently promotes PR elongation at low concentrations (Zhang et al., 2010). Moreover, ABA has an interaction with auxin and ethylene to regulate PR elongation and LR formation negatively (Emenecker and Strader, 2020).

SLs also play a role in regulating RSA

SLs are a class of carotenoid-derived terpenoid lactones initially characterized as germination stimulants for root parasitic plants (Cook et al., 1966) and later identified

as root-derived symbiotic signals for arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005). Subsequent research revealed that SLs have a negative regulatory effect on shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). In *Arabidopsis*, SL biosynthesis starts with DWARF27 (D27) isomerase which converts all-*trans*- β -carotene to 9-*cis*- β -carotene. Then 9-*cis*- β -carotene is catalyzed in the order of CAROTENOID CLEAVAGE DIOXYGENASE 7/MORE AXILLARY GROWTH 3 (CCD7/MAX3) and CCD8/MAX4 to yield carlactone, the last common precursor for SLs (Umehara et al., 2008; Alder et al., 2012). MAX1 converts carlactone to carlactonoic acid which is subsequently methylated by an unknown enzyme to produce methyl carlactonoate (MeCLA) (Abe et al., 2014). MeCLA is mainly catalyzed by LATERAL BRANCHING OXIDOREDUCTASE (LBO) for further non-canonical SL synthesis (Jia et al., 2018). In *Arabidopsis*, SL signaling is mediated by the D14 receptor, together with SCF^{MAX2} protein and a repressor of signaling, D53. SL signaling has a similarity to the auxin signaling pathway, which means the SL/D14/SCF^{MAX2} complex recognizes D53 protein and results in a ubiquitination-dependent degradation of D53. D53 degradation then releases downstream SL signal transduction (Waters et al., 2017). Many roles of SLs have been investigated by numerous studies. SLs are proposed to negatively regulate shoot branching and LR development with the interaction of auxin and cytokinin (Gomez-Roldan et al., 2008; Domagalska and Leyser, 2011; Jiang et al., 2016). SLs have a dual effect on PR elongation that shows promotion at low concentrations and inhibition at high concentrations (Ruyter-Spira et al., 2011). Additionally, SLs promote LR formation in low inorganic phosphate (P_i) conditions and suppress it in high P_i conditions (Ruyter-Spira et al., 2011).

Cytokinins as root development regulators in *Arabidopsis*

Cytokinins are among the most extensively and historically researched phytohormones, playing crucial roles in various aspects of plant biology. The most common types of natural cytokinins include N⁶- (Δ^2 -isopentenyl) adenine (iP), *trans*-zeatin (tZ), dihydrozeatin (DZ) and *cis*-zeatin (cZ). These cytokinins are synthesized from

dimethylallyl diphosphate (DMAPP), which is derived from both the MEP and mevalonate (MVA) pathways. The enzyme adenosine phosphate-isopentenyltransferase (IPT) uses adenosine 5'-phosphates (AMP, ADP, or ATP) to convert DMAPP into iP ribonucleotides (iP RTP, iP RDP, or iP RMP). iP ribonucleotides can be catalyzed by a cytochrome P450 monooxygenase CYP735A to generate tZ ribonucleotides and be consequently converted to tZ riboside or DZ riboside. iP ribonucleotides can also be directly converted into iP riboside. Then these riboside forms of cytokinins (i.e., iPR, tZR and DZR) are converted to active forms of cytokinins such as iP, tZ, and DZ. Meanwhile, cZ is synthesized from tRNA and DAMPP through the action of tRNA-isopentenyltransferase (tRNA-IPT) (Mok and Mok, 2001; Miyawaki et al., 2004; Kurakawa et al., 2007). One of the ways of cytokinin degradation is via cytokinin oxidases (CKXs). CKXs catalyze the irreversible degradation by cleavage of the side chain of cytokinins to produce adenine and adenosine (Werner et al., 2001).

The *Arabidopsis* histidine kinases (AHKs), which are the primary receptors for cytokinins, are primarily localized on the endoplasmic reticulum, as well as on the plasma membrane. When cytokinin binds to AHK proteins, it activates five *Arabidopsis* histidine phosphotransferase proteins (AHP1-AHP5), which shuttle between the cytosol and the nucleus. Cytokinin signaling in *Arabidopsis* is mediated by two groups of *Arabidopsis* Response Regulators (ARR): type-A ARRs and type-B ARRs. The activated AHPs phosphorylate both types of ARRs. Phosphorylation stabilizes type-A ARRs and inhibits the phosphorylation of type-B ARRs, thereby inhibiting cytokinin signaling. Conversely, phosphorylated type-B ARRs bind to DNA, initiating the activation of downstream targets that promote cytokinin signaling (Hwang and Sheen, 2001; Higuchi et al., 2004; To et al., 2004).

Cytokinins are fundamental in promoting cell division and differentiation and influence apical dominance, axillary bud growth, and leaf senescence (Kende and Zeevaart, 1997). Previous work demonstrates that cytokinins act antagonistically with auxin in specifying root stem cells and negatively regulate LR emergence (Müller and

Sheen, 2008). Additionally, cytokinins negatively regulate root apical meristem activity (Werner et al., 2003).

Chromatin remodeling regulation on root development

Chromatin remodeling involves the dynamic modification of chromatin architecture, which allows condensed genomic DNA to become accessible to regulatory transcription machinery proteins, thereby regulating gene expression. This process is often carried out by covalent histone modifications through specific enzymes such as histone acetyltransferases (HATs), deacetylases, methyltransferases, and kinases, or by ATP-dependent chromatin remodeling complexes that facilitate nucleosome movement, ejection, or restructuring (Champoux, 2001; Portela and Esteller, 2010; Furuta et al., 2011).

The role of chromodomain-Helicase-DNA (CHD)-binding proteins in ATP-dependent chromatin remodeling has been studied across numerous species. The CHD3 protein functions as a monomer and can either repress or activate gene expression. Two distinct models have been proposed to explain the regulation of this process. One model suggests that CHD3's suppressive function is accompanied by the accumulation of H3 lysine 27 trimethylation (H3K27me3), a repressive epigenetic mark and that the binding of CHD3 to H3K27me3 is necessary for gene suppression. Another model proposes that the CHD3 protein represses gene expression through the recognition of H3K27me3 (Hu et al., 2014). In plants, the PICKLE protein has been identified as a homolog of the human CHD3/Mi2 ATP-dependent chromatin remodeling factor (Ogas et al., 1997; Ogas et al., 1999). PICKLE is reported to regulate many aspects of plant development, including repression of embryo-specific genes after germination, negative regulation of organ formation in embryonic and post-embryonic development (Ogas et al., 1997; Eshed et al., 1999; Ogas et al., 1999; Ori et al., 2000). Besides, in root development, SUPPRESSOR OF SLR2 (SSR2)/PKL negatively regulates auxin-mediated LR formation in Arabidopsis (Fukaki et al., 2006). CYTOKININ HYPERSENSITIVE 2

604 (CKH2)/PKL has been reported to negatively regulate cytokinin response in callus
605 formation (Furuta et al., 2011).

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Chapter 1

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**Carotenoid metabolism negatively regulates
auxin-mediated primary root growth**

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1.1 Introduction

Carotenoid is essential for photosynthesis. Photosynthesis is the primary pathway for plants to obtain energy. The inhibition of carotenoid biosynthesis enzyme PDS by the herbicide fluridone kills plants under natural conditions. However, under in vitro conditions with sucrose application, fluridone bleaches leaf pigments but does not kill plants, allowing them to develop flowers. In this condition, carotenoid deficiency leads to a reduction of PR growth. This suggests carotenoids play a role in root growth regulation. However, the detailed mechanism is not reported yet. In this study, I identify that carotenoid, as a source of apocarotenoids, play a role in the regulation of root growth mediated by endogenous auxin levels.

1.2 Materials and methods

1.2.1 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. *pds3-1* used in this work refers to *pigment defective 226* (*pde226-1*). The *arf7-1* *arf19-1*, *slr-1*, *yuc1*, *yuc2*, *yuc3*, *yuc4-1*, *yuc5*, *yuc6*, *yuc7*, *yuc8*, *yuc9*, *yuc10*, and *yuc11* plants were described previously (Xu et al., 2017). The *axr1-12* (Lincoln et al., 1990), *aux1-21* (Roman et al., 1995), *aba1-6* (Niyogi et al., 1998), *aba2-1* (González-Guzmán et al., 2002), *psy* (Alonso et al., 2003) and *pde226-1* (McElver et al., 2001) plants were obtained from the Arabidopsis Biological Resource Center. *wei2-1*, *wei7-1*, *wei2-1 wei7-1* (Stepanova et al., 2005) and *sur2* (Delarue et al., 1998) plants were provided from Dr Hidehiro Fukaki (Kobe University). *aba3-2* (Koornneef et al., 1982), *abilc* (Koornneef et al., 1984), *abi3-9*, *abi4-11*, *abi5-7* (Nambara et al., 2002), *ost1-1* (Mustilli et al., 2002) and *pyrQ* (Park et al., 2009) plants were provided from Dr Masanori Okamoto (RIKEN Center for Sustainable Resource Science). *max1-1*, *max2-1* (Stirnberg et al., 2002) and *max3-9* (Booker et al., 2004) plants were provided from Dr Kiyoshi Tatematsu (National Institute for Basic Biology). The *yucca 3 5 7 8 9* (*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. 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 (Murashige and Skoog, 1962; Duchefa Biochemie) supplemented with 1% (w/v) sucrose, 0.6% (w/v) gellan gum, and 0.5 mM MES (pH 5.8). 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Stock solutions of phytohormones and inhibitors were prepared in dimethyl sulfoxide and filtered through a 0.45 μM disc filter.

1.2.2 *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 *A. 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.

1.2.3 *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 chemicals indicated. Plant images were captured using a flatbed scanner (GT-X980 and DS-50000, EPSON) within 4 d after transfer. The LR

density (the number of emerged LR's besides an adventitious root/the root length from the root–shoot junction to the youngest LR toward the root tip) (Dubrovsky and Forde, 2012) and the PR growth rate were analyzed with Fiji software (<https://fiji.sc/>).

1.2.4 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/>).

1.2.5 Quantification of IAA

Seeds were sown in sterilized 300 µm nylon mesh and transferred to control or fluridone plates after 5 d germination together with mesh. Shoot and root samples were harvested at indicated 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 [²H₅]-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).

1.2.6 Bioluminescence imaging analysis

D-Luciferin was purchased from Gold Biotechnology. Four-day-old plants grown on 100 µM D-luciferin medium were transferred to new D-luciferin medium for a 1 d

preincubation and transferred to new D-luciferin medium with or without chemicals indicated. Time-lapse imaging was performed using a camera (ASI294MMPro, ZWO) with control software (SharpCap, v4.0). The images were captured at an interval of 1 h, including 15 min of exposure and 45 min of red and blue light ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) and then saved in a TIFF format for further analysis using Fiji software (<https://fiji.sc/>).

1.2.7 RNA isolation and quantitative reverse transcription polymerase chain reaction analysis

Seeds were sown on control medium with 300 μm nylon mesh. Roots were transferred to mock or 800 nM fluridone medium, and then root samples were harvested at 0, 4, 8, and 24 h and frozen in liquid nitrogen. Total RNA was extracted and purified using an RNA isolation kit (ISOSPIN Plant RNA, NIPPON GENE). Complementary DNA was synthesized from the total RNA according to the manufacturer's instructions (ReverTra Ace qPCR RT Master Mix, Toyobo). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in optical 96-well plates via the LightCycler 480 II system (Roche Life Science) using KOD SYBR qPCR Mix (Toyobo). Primer pairs spanning exon-exon junctions were designed using the QuantPrime program to avoid genomic DNA amplification as listed in Table 1.1 (Xu et al., 2017). The specificity of the reactions was verified via melt curve analysis and capillary electrophoresis (Multina, Shimadzu). Standard curve analysis was used to evaluate the efficiency of the reactions. ACTIN2 was used as an internal standard. The qRT-PCR program included one cycle at 98°C for 2 min, followed by 40 cycles of 98°C for 10 s, 60°C for 10 s, and 68°C for 30 s. The cycle time values were determined using the second derivative maximum method (Tichopad et al., 2003) via LightCycler software (version 1.5, Roche Life Science). The data were analyzed using the comparative threshold cycle (C_t) method ($2^{-\Delta\Delta C_t}$) (Schmittgen and Livak, 2008).

1.2.8 Quantification of carotenoid via high-performance liquid chromatography analysis

Seeds were sown on control medium with 300 µm nylon mesh. The seedlings were grown under the conditions described above. Five days after germination, the seedlings together with nylon mesh were transferred to plates with or without fluridone and with or without 1-naphthaleneacetic acid (NAA) or auxinole or YDF. Shoot tissues (~20 mg) or root tissues (~100 mg) were harvested at specific timepoints and stored at -80°C. Total carotenoids were extracted using acetone wako 1st grade (FUJIFILM Wako Chemicals). Acetone was added to the tissue samples that had been mixed with beads and silica gel and shaken for 2 min. Total carotenoids were separated via centrifugation for 10 min at 14000 rpm at 4°C. High-performance liquid chromatography analysis for carotenoid quantification was performed as previously described (Zapata et al., 2000). An aliquot of the sample was subjected to a C8 column (4.6 × 150 mm, Waters Symmetry C8, Waters) equilibrated with A solution (methanol:acetonitrile:aqueous pyridine solution [0.25 M pyridine] = 2:1:1 [v:v:v]). Pigments were eluted with a linear gradient of 0%–40% B solution (methanol:acetonitrile:acetone = 1:3:1 [v:v:v]) for 2 min, 40%–95% for 6 min, an isocratic elution for 5 min, a linear gradient of 95%–0% for 2 min, and an isocratic elution for 7 min at a flow rate of 1 mL/min. Pigments were identified based on their retention time relative to known standards and quantified by peak area on the chromatogram.

1.2.9 Statistical analysis

The statistical significance is determined by 2-tailed Student's *t*-test (*: $P < 0.05$, **: $P < 0.01$) or one-way ANOVA following Tukey-Kramer test (Different lowercase letters indicate significant differences, $P < 0.05$).

1.3 Results

1.3.1 Fluridone suppresses PDS to inhibit the growth of the primary root

Fluridone demonstrated a dose-dependent suppression of PR growth (Fig. 1.2A, B).

Using time-lapse imaging, the kinetic suppression of PR growth by fluridone was tracked. After fluridone treatment, the root growth of WT was rapidly reduced from about 4 h (Fig. 1.3). Following fluridone treatment of 5-day-old seedlings, the newly emerged true leaves exhibited bleaching, while the established cotyledons showed chlorosis at 3 d after transfer (dat) (Fig. 1.4A). These phenotypes are likely due to reduced carotenoid and chlorophyll levels at 2 dat (Fig. 1.4B–G). No such difference between cotyledons and true leaves was reported in norflurazon-grown or *psy* plants (Van Norman et al., 2014). This suggests that the short-term fluridone treatment may preferentially target developing organs. These results exclude the possibility that the root growth suppression by fluridone is a secondary pleiotropic effect of albinism and reveal a more direct role for carotenoid biosynthesis in PR growth. The LR number in the newly grown areas of PRs after transfer to fluridone medium and the pre-grown areas of PRs were measured. Fluridone induced LR formation in the pre-grown areas but reduced LR number in the newly grown areas (Fig. 1.5). Overall, LR density was increased by fluridone (Fig. 1.2C). Other PDS inhibitors, such as norflurazon, diflufenican and picolinafen, also suppressed PR growth and increased LR density (Fig. 1.6). To investigate the target specificity of fluridone, a fluridone-insensitive trait of PDS (*35S::mHvPDS*) (Arias et al., 2006) was then introduced into the *Arabidopsis* plants. *35S::mHvPDS* plants were confirmed to have some resistance to fluridone in terms of chlorophyll and carotenoid contents (Fig. 1.4B–G) (Arias et al., 2006). Furthermore, *35S::mHvPDS* plants also showed resistance to fluridone in the suppression of PR growth and induction of LR density (Fig. 1.2B, C). These results indicate that PDS is a target of fluridone for the suppression of PR growth and induction of LR formation. PDS is encoded by a single gene, *PDS3*, in *Arabidopsis thaliana* (Qin et al., 2007). The *psy* mutant showed reduced PR growth and LR formation (Van Norman et al., 2014) (Fig. 1.7C). A T-DNA insertion mutant in *PDS3*, *pds3-1* (McElver et al., 2001; Meinke, 2019) also showed reduced PR growth rate and LR density (Fig. 1.8). For both PR growth suppression and LR density induction, *pds3* plants showed insensitivity to fluridone, also indicating that PDS is a target for fluridone (Fig. 1.8 B,

C). Unlike the *pds3* plants, the *psy* plants showed weak resistance to fluridone, and their LR formation was induced by fluridone (Fig. 1.7A, B). These results indicate that the *psy* mutant may not be a null mutant of the *PSY* gene and that the change in root architecture induced by fluridone is specifically dependent on the activity of PDS.

ABA and SLs are β -carotene-derived phytohormones and biosynthesis is suppressed by fluridone (Yoshioka et al., 1998; López-Ráez et al., 2008). It led me to examine ABA- and SL-related mutants for fluridone resistance. If ABA and SLs are response for fluridone-mediated PR growth regulation, their mutants should show higher resistance to fluridone. *ost1* is the mutant of SnRK2.6. *abi1*, *abi3*, *abi4* and *abi5* are downstream ABA response factor mutants. *pyr1pyl1pyl2pyl4* (*pyrQ*) is a quadruple ABA receptor mutant. *max1* and *max3* are SL biosynthesis mutants and *max2* is SL signaling mutant. Neither ABA biosynthesis and signaling mutants nor SL biosynthesis and signaling mutants showed resistance to fluridone (Fig. 1.9A–C). Next, exogenous ABA and *racemic*-GR24 (*rac*-GR24) were employed to complement the suppression of PRs caused by fluridone. The recovery of PR growth was not observed in the fluridone and ABA/*rac*-GR24 co-treated plants compared to fluridone-treated plants (Fig. 1.9G, H). These results indicate that ABA and SLs may contribute less to fluridone-induced PR growth regulation. Since the LR initiation and emergence are under the control of auxin signaling (Du and Scheres, 2018), increased LR density referred me to a direction of the activation of auxin signaling.

1.3.2 Fluridone increases endogenous auxin levels and activates auxin signaling

WT plants exhibited increased IAA levels at 8 and 24 h following fluridone treatment (Fig. 1.10A). However, no significant change in auxin levels was observed in the *35S::mHvPDS* plants (Fig. 1.10B). It indicates PDS inhibition elevates auxin levels. Besides, the elevation of IAA levels by fluridone was not observed in shoots (Fig. 1.10C). To determine the kinetics of auxin signaling, 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. The expression of

DR5:LUC was transiently activated at the root tips regardless of the presence or absence of fluridone, which may be a response to transfer stress (Fig. 1.11). After this transient activation, the *DR5:LUC* expression in fluridone-treated WT plants remained higher than in mock-treated plants after 4 h (Fig. 1.10A). While in *35S::mHvPDS* plants, the *DR5:LUC* expression between mock and fluridone treatment was not different after the initial induction (Fig. 1.10B). These results indicate that the fluridone-activated auxin response, like the induction of endogenous auxin, is dependent on PDS activity. The expression of *DR5:LUC* in fluridone-treated WT plants was reduced to below the mock level after 140 h. This reduction was not observed in *35S::mHvPDS* plants (Fig. 1.11). This data suggests that the temporary inhibition of PDS activity leads to an increase in auxin levels and activation of the auxin signaling pathway, whereas long-term inhibition of PDS activity reduces the auxin response.

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

I 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 and transport were examined for fluridone-mediated PR growth suppression. The PR growth of all the mutants was suppressed by fluridone (Fig. 1.12A). The suppression effect of fluridone in the *AUX/IAA* dominant mutant *aux/iaa19/msg2-1* and *aux/iaa14/slr-1*, and the *ARF* double-mutant *arf7-1 arf19-1* was about 45%, which is similar to WT (55%) (Fig. 1.12A). On the other hand, the PR in the mutant of ubiquitin-activating enzyme E1, *axr1-12* (Lincoln et al., 1990), and the auxin permease mutant *aux1-21* showed less suppression by fluridone (30% and 40%, respectively) (Fig. 1.12A). *axr1-12* and *aux1-7* are reported to show a high level of auxin resistance on root elongation, whereas *msg2-1*, *slr-1*, and *arf7-1 arf19-1* exhibit relatively low or no resistance to auxin on root growth (Timpote et al., 1995; Fukaki et al., 2002; Tatematsu et al., 2004; Okushima et al., 2005). These results suggest that there is a correlation between fluridone resistance and auxin resistance in the auxin mutants.

Optimal levels of auxin are essential for root growth, with both supraoptimal and suboptimal auxin levels reducing root growth (Zhao, 2018). *WEI2* and *WEI7* encode the subunits of anthranilate synthase, which is responsible for the synthesis of tryptophan precursor, anthranilate (Stepanova et al., 2005). The root growth of the *wei2-1 wei7-1* double mutant was insensitive to fluridone, whereas its single mutant showed moderate or no resistance to fluridone, indicating the redundancy between these genes (Fig. 1.12B). The quintuple *YUCCA* mutant, *yucQ*, exhibited a severe root defect due to a reduction in endogenous auxin levels (Chen et al., 2014). Surprisingly, fluridone did not suppress the PR growth in the *yucQ* plants; instead, it promoted it (Fig. 1.12C). This suggests that the inhibition of PDS activity increases endogenous auxin levels (Fig. 1.10A), thereby compensating for the root growth defect in *yucQ* (Chen et al., 2014). None of the *yuc* single mutants showed resistance to fluridone, indicating the redundancy of *YUCCA* genes (Fig. 1.13A, B). These results suggest that PDS activity negatively regulates endogenous auxin levels.

Pharmaceutical modulation of auxin signaling and biosynthesis was coupled with the impact of fluridone treatment. Auxinole, an auxin antagonist targeting the auxin receptor TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) (Hayashi et al., 2012), and yucasin difluorinated analog (YDF), a competitive inhibitor of YUCCA flavin monooxygenase (Tsugafune et al., 2017), were employed in this study. Although auxinole and YDF can alone suppress PR growth, adding fluridone to either inhibitor did not enhance growth inhibition (Fig. 1.12D, E). These results support the hypothesis that fluridone-mediated PR growth inhibition requires auxin signaling and a certain level of endogenous auxin. In addition, exogenous auxin additively suppressed fluridone-induced root growth (Fig. 1.14A), suggesting a direct role of auxin in fluridone-mediated root growth regulation. An inhibitor of auxin efflux transport, N-1-naphthylphthalamic acid (NPA), also suppressed PR growth in a dose-dependent manner (Reed et al., 1998). In contrast to auxinole and YDF, NPA showed an additive effect to that of fluridone in suppressing PR growth (Fig. 1.14B), suggesting that the efflux transporter may not be the primary

target of PDS inhibition.

1.3.4 Auxin negatively regulates carotenoid contents in roots

One of the agricultural applications 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). It is therefore reasonable to consider that carotenoid content is also negatively regulated by auxin signaling. To clarify this hypothesis, the levels of β -carotene in roots were quantified. Auxinole and YDF increased β -carotene levels in the roots and the addition of fluridone reduced them regardless of the presence of auxinole or YDF (Fig. 1.15A, B). The auxin biosynthesis mutants *wei2-1 wei7-1* and *yucQ* increased the levels of β -carotene more than two-fold compared with that in the WT (Fig. 1.15D), while auxin signaling mutants showed insignificant increased β -carotene levels (Fig. 1.15C) which may be due to the redundant functions in these genes. In addition, a negative regulation of carotenoid accumulation by auxin in the shoot tissue was observed (Fig. 1.15E, F). These data suggest that endogenous auxin levels and signaling negatively control β -carotene levels.

1.3.5 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* plants (Fig. 1.16A). Consistently, the expression of the auxin biosynthesis gene *YUC9* increased by ~2.5 times at 4 and 8 h following fluridone treatment (Fig. 1.16B). The induced expression of these genes was associated with the induction time of the *DR5:LUC* expression (Fig. 1.10A). These data suggest that *PDS* inhibition activates auxin biosynthesis and signaling.

The expression of genes in carotenoid biosynthesis (*CAROTENOID CHLOROPLAST REGULATOR2* [*CCR2*]), *CCR1* which regulates the expression of *CCR2* (Ruiz-Sola and Rodríguez-Concepción, 2012), and the carotenoid catabolism pathway (*CAROTENOID CLEAVAGE DIOXYGENASE 1* [*CCD1*], *CCD7* and *CCD8*) (Yuan et al., 2015) (Fig. 1.1) was examined following fluridone treatment. The expression of *CCD7* and *CCD8* was markedly induced at 8 and 24 h in WT plants (Fig. 1.16C, D). In contrast, the expression of *CCR1*, *CCR2* and *CCD1* exhibited minimal induction in comparison to *CCD7* and *CCD8* (Fig. 1.16E–G). These data suggest that after the temporary inhibition of PDS activity, carotenoid catabolism is activated rather than biosynthesis. *CCD7* and *CCD8* have been demonstrated to be induced by auxin (Bainbridge et al., 2005; Hayward et al., 2009). The GENEVESTIGATOR database (<https://genevestigator.com>) (Hruz et al., 2008) also indicates that *CCD7* expression is induced within 0.5 to 1 h following exogenous IAA treatment (Fig. 1.17). These suggest that the increased auxin levels by the inhibition of PDS activity likely feedback to upregulate carotenoid catabolism.

1.3.6 Retinal controls auxin contents in fluridone-mediated PR growth regulation

The target of fluridone was PDS for PR growth inhibition; however, the downstream product of PDS activity responsible for this process remains unknown. Recently, various β -carotene-derived apocarotenoids were reported to be bioactive. D15, an inhibitor of PR growth and LR capacity (Dickinson et al., 2019), has been reported to reduce apocarotenoid contents by inhibiting carotenoid catabolism (Dickinson et al., 2021). Then auxin-related mutants were examined to see whether they showed similar response to D15 as fluridone. D15 suppressed root growth by 17% in WT. In the *axr1-12* mutant, D15 suppressed growth by 9%, which showed no significance to mock-treated plants. In the *msg2-1* mutant, D15 suppressed growth by 19%. In the *wei2-1 wei7-1* mutant, D15 increased growth by 22%, which was not significantly different from mock-treated plants, and root growth in *yucQ* was encouraged by 125% by D15 (Fig. 1.18). These results suggest that although the suppression effect of D15 is weak,

auxin-related mutants show similar response to D15 and fluridone treatment (Fig. 1.12A–C). Thus these results confirm that fluridone-induced increase of auxin levels may be through apocarotenoid biosynthesis. Dickinson et al. (2021) suggest the apocarotenoid retinal as a target of D15 since it rescued D15-induced root growth suppression. Besides, β -cyclocitral is reported to positively regulate root growth and it also complements D15 effect on LR capacity (Dickinson et al., 2019). Thus, β -cyclocitral and retinal were employed as the candidates for the complementation test to fluridone. Retinal, but not β -cyclocitral, completely restored fluridone-induced PR growth inhibition (Fig. 1.19; Fig. 1.20A). An auxin-inducible *pIAA19::Eluc-PEST* reporter (Yamamoto et al., 2017) was used for quantifying auxin signaling. *IAA19* activity increased two-fold in the root tip area following treatment with 10 nM NAA, a synthetic auxin. However, this induction was counteracted by cotreatment with retinal (Fig. 1.20E). These data suggest that exogenous retinal has an antagonistic effect on auxin. If true, retinal may complement the inhibitory effect of auxin. Indeed, retinal restored NAA-induced PR growth inhibition (Fig. 1.20B). Although retinal did not affect PR growth in the WT, PR growth in the *wei2-1 wei7-1* and *yucQ* mutants was significantly decreased (Fig. 1.20C). Furthermore, retinal demonstrated an antagonistic effect on auxin-induced LR formation in the presence of NAA and tryptophan (Fig. 1.21D, E). The high LR density of *sur2* was also reduced by retinal (Fig. 1.21G). These data are consistent with a negative regulation of endogenous auxin levels by retinal. However, no strong reduction but an induction in IAA levels was detected in the roots of the retinal-treated plants (Fig. 1.21I). Auxin catabolism inhibitor KKI (Fukui et al., 2022) inhibits PR growth in a dose-dependent manner (Fig. 1.20D) like as treatment with exogenous auxin (Fig. 1.21C). Interestingly, retinal did not rescue the KKI-induced suppression of PR growth and induction of LR formation (Fig. 1.20D; Fig. 1.21H). It suggests that endogenous auxin increase by KKI overcomes the antagonistic effect of retinal on auxin. Although the retinal levels in fluridone-treated plants remain to be determined, these results indicate that retinal may accelerate auxin catabolism to control endogenous auxin levels.

1.4 Discussion

1.4.1 Inhibition of PDS activity increases the levels of auxin in roots

In this study I aim to investigate the regulation of root growth by temporary inhibition of carotenoid biosynthesis. Here I demonstrated that the temporary application of fluridone increased endogenous auxin levels specifically in roots but not in shoots (Fig. 1.10A, C). Such a temporary inhibition of PDS activity may exceed the optimal concentration of auxin levels for root growth, thereby resulting in the suppression of PR growth and the promotion of LR formation (Fig. 1.2B; Fig. 1.10A). The suboptimal endogenous auxin levels observed in *yucQ* mutants, known for their short roots, could be partially restored by low-dose exogenous IAA application (Chen et al., 2014). The root-specific increase in auxin levels induced by PDS inhibition correlates with the partial recovery of root growth observed in *yucQ* mutants (Fig. 1.11C). The ectopic expression of *YUC3* in the shoot of the *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). Correspond with it, fluridone did not affect IAA levels in shoots (Fig. 1.10C). The *sur2/fsp1* and *yuc1D* plants accumulate endogenous auxin and increase the growth of hypocotyls (Delarue et al., 1998; Zhao et al., 2001). However, the length of hypocotyls was similar between fluridone-treated and mock-treated plants (data not shown), confirming a root-specific induction of auxin by fluridone. Therefore, the effect on root growth of fluridone in the WT and *yucQ* is root-specific and not based on shoot-derived auxin. There is another question: both the *yucQ* mutant and YDF treatment reduce PR growth; however, fluridone encouraged root growth in the *yucQ* mutant but had no further suppression or recovery to YDF treatment (Fig. 1.12C, E). One possibility is that auxin biosynthesis in *yucQ* is not completely abolished, allowing fluridone to induce auxin within suboptimal levels, consequently inducing root growth. However, a high dose of

YDF completely blocks root local auxin biosynthesis from the IPyA pathway, thus fluridone cannot elevate endogenous auxin levels to suppress root growth. To address it, the endogenous IAA levels of root tissues in *yucQ* and YDF-treated plants after fluridone treatment are necessary in the future. Another hypothesis is that fluridone inhibits enzymes involved in the IG biosynthesis pathway, such as SUR1 and SUR2. In this hypothesis, fluridone reduces SUR1 or SUR2 activity to overflow auxin biosynthesis from the IPyA pathway in *yucQ* plants; however, this overflow is abolished by YDF which inhibits YUCCA activities. The genetical and pharmaceutical approaches should be performed in this pathway in the future. Like the *yucQ* plants, the *wei2 wei7* plants showed a reduction in endogenous IAA levels and a short root phenotype. Although the root of *wei2 wei7* showed resistance to fluridone, it did not restore the root growth of *wei2-1 wei7-1*, in contrast to that of *yucQ* (Fig. 1.12B, C). A low-dose application of anthranilic acid, a precursor of IAA and tryptophan, partially recovered the root growth in *wei2-1 wei7-1* without rescuing its endogenous IAA levels (Doyle et al., 2019). This suggests that the short roots of the *wei2-1 wei7-1* plants require anthranilic acid rather than increasing the suboptimal concentration of IAA to compensate for their growth. In fact, a higher concentration of anthranilic acid significantly but not completely rescued the root growth of *wei2-1 wei7-1* (Doyle et al., 2019). Low-dose application of exogenous NAA did not rescue or inhibit root growth in *wei2-1 wei7-1* (Fig. 1.13C), further supporting that endogenous auxin is suboptimal and it is not a primary requirement for rescuing the PR growth in *wei2-1 wei7-1*. In contrast to auxinole and YDF, exogenous auxin and fluridone exhibited an additive effect on PR growth suppression (Fig. 1.14A). This suggests that the optimal level of auxin and auxin signaling are required for PR growth and that excessive or insufficient levels of auxin can reduce PR growth.

1.4.2 The temporary inhibition of PDS activity preferentially affects newly synthesized carotenoids, with a minor impact on the carotenoid pool

Carotenoid biosynthesis largely affects chlorophyll biosynthesis and chloroplast

development (Qin et al., 2007; Kim and Apel, 2013). In this work, I showed that when seedlings with established cotyledons were exposed to fluridone, the newly emerged true leaves were bleached, while the established cotyledons displayed chlorosis (Fig. 1.4A). This phenotype differs significantly from the albino phenotype observed in the carotenoid-deficient mutants (i.e., *pds3* and *psy*) (Fig. 1.7C; Fig. 1.8A), or in the seedlings grown on PDS inhibitor media (Van Norman et al., 2014). The application of norflurazon solution to 5-day-old seedlings causes leaf chlorosis and disrupts the chloroplast integrity (Kim and Apel, 2013). Moreover, the application of fluridone to maize seedlings causes the newly formed leaf tissues to become white (Kim et al., 2004). These are consistent with my data and suggest that the temporary inhibition of PDS activity preferentially affects newly synthesized carotenoids. Carotenoids in mature chloroplasts and chromoplasts are more stable than those newly synthesized (Merzlyak and Solovchenko, 2002; Ruiz-Sola and Rodríguez-Concepción, 2012). PDS proteins may be involved in the trafficking route of carotenoid transport from the synthesis sides (envelope membranes) to the functional sides (thylakoid membranes) (Lopez et al., 2008). Thus, it is reasonable to assume that the carotenoid synthesis and transport in new organs are more easily inhibited by fluridone. While the chlorophyll and β -carotene contents are less than 4% in the seedlings grown on fluridone-containing medium compared to mock medium (Bartels and Watson, 1978), the remaining contents of fluridone-treated plants were 50% of chlorophylls and 25% of β -carotene compared to control (Fig. 1.4B, C, G), further supporting this hypothesis.

1.4.3 The roles of retinal on fluridone-mediated root growth

Dickinson et al. (2021) report that retinal (1 μ M) induces LR capacity. In this study, the application of a higher dose of retinal (20 μ M) showed antagonistic effects to auxin on the PR growth and the LR formation (Fig. 1.20B, E; Fig. 1.21C, D, E, G). Although retinal partially compensated for the short roots of the *pds3-1* plants (Fig. 1.20B), it did not alter the density of LRs (Fig. 1.21D). Therefore, it is speculated that the retinal-induced reduction of auxin response postpones the emergence of LRs. As evidence, the

total length of PR in mock and retinal-treated plants was comparable, while retinal-treated plants exhibited a shorter LR branching zone (from the root–shoot junction to the youngest LR toward the root tip) and a longer no LR zone (from the youngest LR toward the root tip to the root tip) compared to mock plants (Dubrovsky and Forde, 2012) (Fig. 1.21F). This also provides an explanation for the different effects of retinal on LR formation in this work and previous research. It should be noted that Dickinson et al. (2021) measure the LR capacity by excising the root tips to encourage LR formation which evokes the root-cut response (Xu et al., 2017), which differs from this study. The severe short root phenotype in *yucQ* plants is further repressed by YDF due to further inhibition of auxin biosynthesis in *yucQ* plants (Tsugafune et al., 2017). Retinal also suppressed the root growth of *yucQ* plants (Fig. 1.20C); however, it did not alter endogenous free IAA levels of WT in the observation periods of this study (Fig. 1.21I). This indicates that the modulation of auxin levels by retinal may occur rapidly and the quantification in shorter periods is necessary to observe it. To address it, a shorter time course in IAA quantification by retinal treatment is necessary in the future. Also the reason why retinal alone cannot reduce endogenous auxin levels but induces them may be due to the negative feedback from reduced auxin levels since KKI is reported to transcriptionally down-regulate the auxin biosynthesis gene *YUC6* (Fukui et al., 2022). Instead, the antagonistic effect of retinal on auxin was abolished by the auxin catabolism inhibitor KKI (Fig. 1.20D; Fig. 1.21H), suggesting that retinal may target downstream substrates of GH3 in the auxin catabolic pathway. To confirm that KKI-induced increases in auxin levels are not reduced by retinal, it is necessary to measure endogenous IAA levels following co-treatment with NAA and retinal, as well as KKI and retinal. The study of mutants in the auxin catabolic pathway on fluridone and retinal may be required for this confirmation. The expression of *YUC9* was enhanced by fluridone treatment (Fig. 1.16B). It may suggest that the fluridone-induced accumulation of endogenous IAA may be primarily contributed by the suppression of the auxin catabolic pathway rather than by the activation of the auxin biosynthesis pathway. This induction also shows the possibility that retinal targets the IG

1079 biosynthesis pathway, which reduces tryptophan substrate for IAA biosynthesis.

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1081 *1.4.4 The difference between continuous carotenoid deficiency and temporary*
1082 *inhibition of PDS activity*

1083 Van Norman et al. (2014) and this study showed that both the *psy* and *pds3-1* plants had
1084 shorter PR lengths compared to WT (Fig. 1.7A, C; Fig. 1.8A, B). Similarly, temporary
1085 exposure to fluridone reduced PR growth. Short-term fluridone treatment led to rapid
1086 and modest suppression of root growth, whereas long-term exposure (>60 h) resulted
1087 in accelerated suppression of root growth (Fig. 1.3), which may correspond to the short
1088 PRs in *psy* and *pds3-1*. In fact, retinal completely recovered the root growth in the
1089 fluridone-treated WT plants on the second day after treatment (Fig. 1.22). However, on
1090 the third day after treatment, retinal partially rescued fluridone-induced root growth
1091 suppression, while the root growth of *pds3-1* was continuously promoted by retinal (Fig.
1092 1.22), suggesting that the short-term (2 dat) and long-term (3 dat) fluridone treatment
1093 suppresses root growth independently, and the former results from blocking retinal
1094 biosynthesis. The temporary fluridone application resulted in a degradation of the
1095 mature plastids which shows chlorosis in established cotyledons and bleaching in newly
1096 emerged leaves (Fig. 1.7A), while the plastids of the albino *pds3* mutant lose the ability
1097 to differentiate (Qin et al., 2007). This discrepancy in root growth recovery may indicate
1098 that the degradation of plastids and the undifferentiated proplastids suppress the root
1099 growth independently.

1100 Although both *psy* and *pds3-1* mutants exhibited a reduction in the number of LRs
1101 (Fig. 1.7A; Fig. 1.8C), fluridone treatment showed contrasting effects on LR formation
1102 along the PRs. Specifically, fluridone increased LR formation in the pre-grown areas of
1103 PRs but decreased it in the newly grown areas of PRs in WT plants (Fig. 1.5). Previous
1104 studies have reported an induction of LR formation by fluridone in excised tomato roots
1105 (Hooker and Thorpe, 1998). It is hypothesized that the process of plastid degradation
1106 leads to increased auxin levels and consequent induction of LR formation in pre-grown
1107 roots, while newly grown roots, characterized by undifferentiated proplastids, exhibit

reduced LR formation, akin to the phenotype observed in *psy* and *pds3-1* mutants (Fig. 1.7A; Fig. 1.8C) (Van Norman et al., 2014). This hypothesis is supported by the observation that the *DR5:LUC* expression at the root tips increased upon fluridone application but eventually decreased with long-term incubation (Fig. 1.11A).

1.4.5 Putative model for carotenoid-mediated root growth

In this study, I found that the apocarotenoid retinal controls the growth of PRs through regulating endogenous auxin levels (Fig. 1.23). Temporary inhibition of PDS activity blocks retinal biosynthesis and then leads to auxin accumulation to suppress root growth. Long-term exposure to fluridone further suppresses root growth, with the underlying mechanisms yet to be elucidated. The role of retinal here may be different from that of promoting LR capacity (Dickinson et al., 2021). There are two possible pathways for retinal to regulate auxin contents and response. First is that retinal may accelerate IAA catabolism to reduce endogenous auxin levels. Second is that retinal may up-regulate genes or proteins in IPyA-independent auxin biosynthesis pathway (e.g., IG biosynthesis), such an activation of IPyA-independent pathway decreases the precursors of auxin (e.g., tryptophan). In addition, auxin may further regulate carotenoid accumulation through a feedback mechanism involving the expression of genes associated with carotenoid catabolism. This model provides insight into the role of the apocarotenoid retinal in root growth regulation. The quantitative analysis of retinal and its derivatives, as well as their targets that may regulate auxin homeostasis, is warranted in the future.

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Chapter 2

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The general examination of root-cut response regulation factors

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2.1 Introduction

Quick organ regeneration is one of the ways for plants to adapt to the environment. When a portion of the PR is removed or damaged by physical obstacles or animal consumption, it will disrupt plant development or even pose a threat to plant growth. However, root system has a remarkable ability for regeneration (Al-Ghazi et al., 2003; Ditengou et al., 2008; Sena and Birnbaum, 2010; Sugimoto et al., 2011). Additionally, there is an agricultural technique known as root pruning, which utilizes the regeneration ability of plants. The removal of a portion of the root system by root pruning allows the plant to regenerate a new root system with a smaller size and a greater number of branches in the remaining roots (Wang et al., 2014). LR growth induced by mechanical root damage has been documented since long ago. The first report of root regeneration by root-cut is in *Vitis sp.* (grape) (Zimmerman and Hitchcock, 1935). Root pruning of citrus has been reported to promote flowering and the growth of a more fibrous root system (Budiarto et al., 2019). Also root pruning increases the growth and above-ground biomass in white cabbage (Báth et al., 2008). While root pruning is generally applied as an agricultural technique, the removal of a part of PR under sterile conditions reflects the result of root pruning. The regeneration response to root-cut, addressed as Root-Cut-induced LR Number (RCN) has been partially elucidated (Xu et al., 2017). However, the mechanisms of Root-Cut-induced LR Growth (RCG) are still largely unknown. In this study, I try to investigate the factors in the regulation of LR growth by root-cut. I identified cytokinins as the negative regulators for LR growth. I also investigated the regulatory role of fluridone and carotenoid metabolism substrates in LR growth regulation.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Arabidopsis mutants and WT plants used in this study were in the Columbia background. *35S::AtCKX1* and *35S::AtCKX2* (Werner et al., 2001) plants were provided by Dr Thomas Schmülling (FREIE UNIVERSITÄT BERLIN). *pkll-1* (Ogas

et al., 1997) plants were obtained from the Arabidopsis Biological Resource Center. *ckh2-3*, *pkr2-2* and *ckh2-3 pkr2-2* (Furuta et al., 2011) were provided by Dr Tatsuo Kakimoto (Osaka University). Seeds were surface-sterilized with chlorine gas for at least 45 min. seeds were suspended in 0.35% agarose and sown on half-strength MS medium (Duchefa Biochemie) supplemented with 1% (w/v) sucrose, 0.6% (w/v) gellan gum and 0.5 mM MES pH 5.8. 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 conditions (80-110 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Stock solutions of inhibitors were prepared in dimethyl sulfoxide and filtered through a 0.45 μm disc filter.

2.2.2 Root cutting and the quantification of LR numbers and LR growth rate

Plants were grown on vertical plates for 4 d, then transferred to new half-strength MS medium with or without inhibitors. After 1 d of preincubation, the root was cut 12mm from the root-shoot junction. Plant images were acquired with a flatbed scanner (GT-X980, EPSON) 4d after root-cutting. RCN was observed at 4 d after root-cut (dac). The growth rate of the 1st LR was observed between 2 dac and 3 dac. The number of emerged LRs within 12mm from the root-shoot junction, as well as the length of PR and first emerged LR were analyzed with Fiji software (<https://fiji.sc/>).

2.2.3 Quantification of cytokinins

Seeds were sown in sterilized 300 μm nylon mesh and transferred to mock or fluridone plates after 4 d germination together with mesh. The PRs were cut after 1 d preincubation. Root samples were harvested at indicated timepoints after root-cut and frozen in liquid nitrogen. Hormonome analysis was conducted as previously described (Xu et al., 2017). Briefly, samples of ~100 mg Fw were suspended in 80% (v/v) aqueous methanol with [²H₅]-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).

2.2.4 Statistical analysis

The statistical significance is determined by 2-tailed Student's *t*-test (*: $P < 0.05$, **: $P < 0.01$) or one-way ANOVA following Tukey-Kramer test (Different lowercase letters indicate significant differences, $P < 0.05$).

2.3 Results and Discussion

2.3.1 Fluridone specifically suppresses root-cut-induced LR growth via the auxin pathway

To confirm the effects of root regeneration by root-cut, the PRs of 5-day-old plants were cut at 12 mm from the root–shoot junction (RSJ). The LR number on the remaining 12 mm root section in root-cut plants increased compared to the corresponding areas in intact plants at 4 dac (Fig. 2.1A). Besides, the 1st LR in cut plants was longer than that in intact plants at 3 dac. By the measurement of growth in 1st LR between cut and intact plants, I found that the growth of 1st LR in cut plants was much quicker than that in intact plants (Fig. 2.1B). These results confirm RCN and RCG which is analyzed in the previous research (Xu et al., 2017). In this study, I focus on RCG.

The role of fluridone in regulating PR growth, as discussed in Chapter 1, prompted an investigation into its effects on RCN and RCG. On mock treatment, the LR number and growth of 1st LR in cut plants was quicker and more than those in intact plants, resulting in RCN and RCG (Fig. 2.2A, B). First I examined RCN under fluridone condition. Root-cut induced the LR formation in both mock- and fluridone-treated plants. Overall, RCN persisted with the increased dose of fluridone up to 800 nM (Fig. 2.2A). Regarding RCG, by the increasing concentration of fluridone, the growth of 1st LR in intact plants was not altered much, but that in cut plants was reduced in dose dependent manner. As a result, RCG was reduced and abolished at an 800 nM fluridone

concentration (Fig. 2.2B). These results indicate that fluridone is a specific inhibitor for RCG. It also indicates that root-cut triggers an unknown pathway to induce LR growth, and the effect of this unknown pathway may be inhibited by fluridone. Moreover, RCN in *35S::mHvPDS* was not affected by fluridone, which is the same as WT (Fig. 2.2C). On the other hand, *35S::mHvPDS* plants showed resistance to fluridone in RCG (Fig. 2.2D). These results suggest that fluridone suppresses RCG via inhibiting PDS activity. Since fluridone is found to induce endogenous auxin levels in Chapter 1 (Fig. 1.10A), then I wonder whether fluridone suppresses RCG via increasing auxin levels. First I treated plants with dose concentrations of NAA. Results showed that root-cut-induced LR growth was reduced in dose dependent manner while that in intact plants was affected less upon to 80 nM NAA (Fig. 2.3A). And RCG was abolished at 320 nM NAA (Fig. 2.3A). This result was similar to fluridone treatment (Fig. 2.2B). Then I employed auxin signaling and transport mutants used in Chapter 1 and treated them with fluridone (Fig. 1.12A). In mock condition, these mutants showed RCG (Fig. 2.3B), indicating that root-cut can overcome abnormal auxin signaling to encourage LR growth. Interestingly, RCG still existed in these mutants even under fluridone treatment (Fig. 2.3B). This result suggests that fluridone may suppress RCG via the auxin signaling pathway.

2.3.2 Apocarotenoids are responsible for fluridone-mediated RCG abolishment

Then I explore the downstream substrates that participated in fluridone-mediated RCG regulation. In Chapter 1, I investigated that the apocarotenoid retinal is responsible for fluridone-mediated PR growth regulation (Fig. 1.23). Hence, I employed retinal as well as β -cyclocitral and β -ionone for the complementary experiment with fluridone. Fluridone treatment alone abolished RCG; however, co-treatment of fluridone and apocarotenoids partially recovered LR growth by root-cut (Fig. 2.4), suggesting that fluridone reduces root-cut induced LR growth partially via reducing apocarotenoid contents. The recovery of RCG with retinal treatment also suggests that the fluridone-mediated regulation of LR growth may be also related to the auxin pathway. However,

β -cyclocitral is excluded from the regulation of fluridone-mediated PR growth in Chapter 1 (Fig. 1.19) but shows participation in fluridone-mediated RCG regulation (Fig. 2.4A), suggesting that the regulation mechanisms between PR and LR growth may differ from each other, and the fluridone-mediated regulation of RCG may be regulated by other factors besides of auxin.

2.3.3 Root-cut reduces endogenous cytokinin levels

Xu et al (2017) reported that root-cut elevates endogenous IAA levels in roots, and the increase of auxin is the key regulator for RCN. However, the apocarotenoid complementary experiment referred the unknown factors participating in this process. Thus I tried to investigate the RCG regulator from the content modulation of phytohormones in roots after fluridone treatment and root-cut. Besides the levels of endogenous IAA, which is elevated shown in Chapter 1 (Fig. 1.10A), there is no significant modulation of phytohormones by fluridone treatment examined in this study (e.g., jasmonic acid, jasmonic acid isoleucine and cytokinins) (data not shown). Surprisingly, the endogenous levels of tZR, the precursor of tZ-type cytokinin, was decreased after root-cut (Fig. 2.5). Moreover, this reduction of cytokinin levels also occurred in fluridone-treated plants (Fig. 2.5). The reduction of endogenous cytokinin levels by root-cut suggests that cytokinins may play a role in RCG regulation, and root-cut-induced cytokinin reduction may act upstream of fluridone inhibition.

2.3.4 Cytokinins play a negative role in the regulation of LR growth

CKXs encode cytokinin oxidases which convert active forms of cytokinins to cytokinin metabolite adenine and adenosine (Werner et al., 2001). The role of cytokinins in LR growth has been reported in *Hordeum vulgare* (barley) that the reduction of cytokinin levels increases the total root length in barley plants (Ramireddy et al., 2018). Overexpression of AtCKX1 and AtCKX2 transgenic tobacco plants have longer PR and LR length (Werner et al., 2001). However, the cytokinin role of LR growth in *Arabidopsis* is less elucidated yet. Thus I employed the overexpression of AtCKX1 and

AtCKX2 transgenic plants (*35S::AtCKX1* and *35S::AtCKX2*) to examine RCG. The LR growth of *35S::AtCKX1* in intact plants was much quicker than that of WT, and root-cut could not further enhance it (Fig. 2.6A). It indicates that root-cut induces LR growth via reducing endogenous cytokinin levels. However, the LR growth in *35S::AtCKX2* plants was in the same level in intact plants, but root-cut did not enhance its growth like as *35S::AtCKX1* (Fig. 2.6A). It may be explained that the overexpression of *AtCKX2* is not as strong as *AtCKX1* so that the cytokinin levels in *35S::AtCKX2* are not as low as in *35S::AtCKX1*. The quantification of endogenous cytokinin levels in these two transgenic plants is necessary in the future. Then I treated *35S::AtCKX1* with fluridone to examine whether fluridone suppresses RCG via the cytokinin pathway. Fluridone treatment largely reduced LR growth in both intact and cut plants to the same level. However, the LR growth in *35S::AtCKX1* was quicker than that in WT after fluridone treatment (Fig. 2.6B). Since there is no change in cytokinin levels after fluridone treatment, fluridone may activate cytokinin signaling to suppress root-cut-induced LR growth. Also there is a possibility that cytokinins and fluridone regulate RCG independently. Further examination is needed in the future.

2.3.5 Chromatin remodeling factor *PICKLE* is necessary for RCG

The regulation of PKL in LR growth is less studied yet. Since PKL/SSR2/CKH2 has been reported to regulate auxin-mediated LR formation and cytokinin-mediated callus formation, mutations in *PKL* were examined for RCG. I examined *pkll-1*, *ckh2-3* (an allele of *pkll* mutant), *pickle-related 2* (*pkr2-2*) and *ckh2-3 pkr2-2* double mutant. Results showed that *pkll*, *ckh2-3* and *ckh2-3 pkr2-2* abolished RCG, but not for *pkr2-2* (Fig. 2.7). It indicates that *PICKLE* is necessary for RCG. Interestingly, the LR growth of *pkll*, *ckh2-3* and *ckh2-3 pkr2-2* in intact plants was slightly quicker than that of WT (Fig. 2.7), which corresponds to the result of *35S::AtCKX1* (Fig. 2.6A). This result suggests that *PICKLE* may negatively regulate cytokinin signaling to control LR growth.

2.3.6 Putative model of fluridone- and cytokinin-mediated RCG regulation

As shown in Fig. 2.8, root-cut likely reduces endogenous cytokinin levels to enhance LR growth. Reduction of cytokinin levels by overexpression of *AtCKX1* or downregulation of cytokinin response by PKL/CKH2 leads to an acceleration of LR growth which cannot be enhanced by root-cut. Root-cut may also possibly up-regulate *AtCKX1* to reduce endogenous cytokinin levels. On the other hand, fluridone specifically suppresses root-cut-induced LR growth via the auxin pathway. This suppression could be partially rescued by β -cyclocitral and β -ionone, indicating the participation of apocarotenoids in fluridone-mediated RCG regulation. However, it is still unclear whether there is an interaction between cytokinin and the carotenoid metabolism pathway and need to be clarified in the future.

General Discussion

The root system is very important for plants in the aspect of live evolution, since it is the adaptation for plants to live on lands. The existence of roots allows plants to anchor into soil and absorb water and nutrients. The development of the root system, including the elongation of PRs, and the branching and elongation of LR, is the major strategy for plant survival. Plants always tend to elongate longer PRs and branch more LR to absorb water and nutrients more effectively. The regulation of RSA is always the subject of the research field. Carotenoids are the ancient and universal metabolites for the live species on the Earth. Besides of their roles on photosynthesis, they also serve as the precursors of apocarotenoids, which support for live activities. The apocarotenoid retinal in animals is as the precursor of vitamin A and necessary for antioxidation and vision system. However, its role on plant development, especially for RSA regulation, is still largely unknown. In this study, the role of apocarotenoids in the regulation of RSA, including auxin-mediated primary root growth and RCG, are investigated.

In the first chapter of this study, I examined the role of retinal on regulating PR growth, one of the aspects of RSA. Inhibition of β -carotene biosynthesis with the 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 *pLAA19::Eluc-PEST*, indicating an antagonistic effect of retinal on endogenous auxin

1357 levels. Interestingly, the auxin-deficient mutants *wei2 wei7* and *yucQ* accumulated more
1358 β -carotene compared with the wild type. The expression of *CCDs*, which converts β -
1359 carotene into apocarotenoids, is auxin-inducible and increases following fluridone
1360 treatment. These results indicate feedback regulation during apocarotenoid biosynthesis
1361 in plant tissue. Thus, I conclude apocarotenoid retinal regulates auxin levels and
1362 response.

1363 In the second chapter, I examined the role of apocarotenoids on RCG. I found that
1364 fluridone suppresses RCG partially by reducing apocarotenoid contents. This
1365 suppression may be mediated by the auxin signaling pathway. Besides, cytokinins play
1366 a negative role in RCG regulation, and PKL may regulate RCG via the cytokinin
1367 signaling pathway.

1368 The apocarotenoids as the metabolites of carotenoids have been identified their roles
1369 on plant development, especially for their bioactive functions in recent years. In this
1370 study, the bioactive roles of retinal, such as the regulation of PR and LR growth, are
1371 identified. These findings refer a high possibility that apocarotenoids may also affect
1372 the morphogenesis of other live species. For example, how apocarotenoids regulate the
1373 morphology of moss plants, whether retinal controlling auxin levels occurs only in
1374 *Arabidopsis* or it also happens in moss plants. These questions may be a good subject
1375 for research in the future. Back to this study, how retinal controls endogenous auxin
1376 levels and the interaction between the apocarotenoid and cytokinin pathway in RCG
1377 regulation are still in mystery. The study of controlling auxin levels by retinal and RCG
1378 regulation by cytokinins will be my future interest.

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I also would like to thank my subsupervisors, Prof. Tomomichi Fujita and Prof. Ryohei Nakano for giving me valuable suggestions and comments during my revision of thesis. Here I will put the major concerns and the response toward these comments during the revision of thesis as below.

The Major Concerns for both subsupervisors:

1: The numbering of Figures should be reconsidered.

Answer: Thank you very much for this comment. After revision, I rearrange the figure number in the order of appearance.

2: Forms of figure representation should be uniform.

Answer: Thank you very much for this comment. After revision, the graphs of PR growth are shown with absolute value, except for Fig. 1.22, which is compared to mock-treated WT plants and take the value from 3 independent biological replicates. And in the comparison of two values, I additively add percentage above the bars to show the relative root growth compared to non chemical-treated plants.

3: The Discussion of Chapter 2 is weak and it is better to add crosstalk within Chapter 1 and 2.

Answer: Thank you very much for this comment. I have rearranged the contents of Chapter 2 with a more logical way and add discussion into Results to combine as “Results and Discussion”. I add some crosstalk between Chapter 1 and 2 in the part “Results and Discussion” and “General and Discussion” part.

The Concerns for Prof. Fujita:

1: Line 197-209, the link between carotenoid and root is weak, and a reason to link them is necessary.

Answer: Thank you very much for this comment. I revised abstract at all and stated the link between carotenoids (apocarotenoids) and root development (line 191-217).

2: Line 254, Abbreviation, AUX1, AUXIN RESISTANT 1 wrong.

Answer: Thank you very much for this comment. I delete it.

3: Line 328 “the short-term application” how short period indicates.

Answer: Thank you very much for this comment. I plan to state as application of carotenoid biosynthesis inhibitors after 5-day-old seedlings, and within a short-term period they suppress RSA which indicates before 60 h indicated later in Discussion. Finally I delete this statement.

4: Line 405 can not see branch of α - and β -carotene from Fig. 1.1.

Answer: Thank you very much for this comment. After revision, I add the branch of α - and β -carotene in Fig. 1.1.

5: Line 463, should mention about lateral bud emergence as SL function.

Answer: Thank you very much for this comment. After revision, I add reference mentioned about SL function of lateral bud emergence “Subsequent research revealed that SLs have a negative regulatory effect on shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008).” (line 525-526).

6: Line 557, more detailed introduction is recommended.

Answer: Thank you very much for this comment. “Carotenoid is essential for photosynthesis. Photosynthesis is the primary pathway for plants to obtain

energy. The inhibition of carotenoid biosynthesis enzyme PDS by the herbicide fluridone kills plants under natural conditions. However, under in vitro conditions with sucrose application, fluridone bleaches leaf pigments but does not kill plants, allowing them to develop flowers. In this condition, carotenoid deficiency leads to a reduction of PR growth. This suggests carotenoids play a role in root growth regulation. However, the detailed mechanism is not reported yet. In this study, I identify that carotenoid, as a source of apocarotenoids, play a role in the regulation of root growth mediated by endogenous auxin levels.” (line 626-634) I think this introduction is more specific to match to this study.

7: Line 730 “rapid response” wonder whether it can be said “rapid” or not.

Answer: Thank you very much for this comment. I think the root growth suppression within 4 h is very quick.

8: Line 741, GR24 5 μ M has recovery of PR growth under fluridone condition (Fig. 1.8H).

Answer: Thank you very much for this comment. If SLs mainly contribute to fluridone-mediated PR growth suppression, they should recover root growth compared to fluridone-treated plants. The recovery by cotreatment of SL and fluridone compared to SL treatment may indicate that fluridone reduce the effect of root growth suppression by high dose of SLs. After revision I replace graph with fluridone and fluridone+GR24 treatment (Fig. 1.9H)

9: Line 760, the *DR5:LUC* expression of *mPDS* plants in fluridone treatment was higher than mock treatment at 0-6 h.

Answer: Thank you very much for this comment. The initial induction of *DR5:LUC* expression may be caused by transfer stress, indicated in line 825-826.

1493 **10:** Line 790 “severe root defect”.

1494 **Answer:** Thank you very much for this comment. In graph it is not shown the severe
1495 root growth of *yucQ* plants. 60% root growth is the root growth of WT under
1496 fluridone condition compared to mock-treated WT plants. With revision, I
1497 put absolute root growth of WT and *yucQ* plants (Fig. 1.12C).

1498

1499 **11:** Line 861-863, what does it means.

1500 **Answer:** Thank you very much for this comment. I planned to mention it to show the
1501 difficulty of complementary experiment to fluridone using β -carotene which
1502 can confirm the direct participation of the carotenoid pathway in fluridone-
1503 mediated root growth regulation. Finally I delete it.

1504

1505 **12:** Line 887, at 8 h significant reduction of auxin levels by retinal treatment was
1506 observed.

1507 **Answer:** Thank you very much for this comment. After revision, I state it as “no strong
1508 reduction but an induction in IAA levels was detected in the roots of the
1509 retinal-treated plants (Fig. 1.21I)” (line 960).

1510

1511 **13:** Line 943, root-specific, what kind of system exist.

1512 **Answer:** Thank you very much for this comment. Local auxin biosynthesis is crucial
1513 for root growth, and local auxin biosynthesis of roots in *yucQ* mutant recovers
1514 its root growth but shoot-derived auxin contributes less (Chen et al., 2014).
1515 Thus I conclude that fluridone increases auxin contents in roots which is
1516 important for root growth.

1517

1518 **14:** Line 1068 explain the background of the apocarotenoid retinal roles for root growth
1519 regulation.

1520 **Answer:** Thank you very much for this comment. In fact, the role of retinal in higher
1521 plants is less studied yet. the only report is the role of retinal in periodic LR

1522 patterning (Dickinson et al., 2021).
1523
1524 **15:** Chapter 2 comments.
1525 **Answer:** Thank you very much for the comments in Chapter 2. After revision, I
1526 rearranged the contents in Chapter 2. Thus the comments for Chapter 2 are
1527 mainly abolished. For line 1236 “*pkl* mutant data”, I deleted the figure of LR
1528 distribution.
1529
1530 **16:** Figure 1.2, “PR growth was analyzed from 2 dat to 3 dat” need more precise time
1531 to harvest?
1532 **Answer:** Thank you very much for this comment. I think the root growth between 2
1533 and 3 d after transfer is reasonable since the effect of fluridone is obvious and
1534 is suitable for observing recovery in complementary experiment.
1535
1536 **17:** Figure 1.14 n.d. should be excluded from statistical analysis.
1537 **Answer:** Thank you very much for this comment. After revision, I excluded n.d. data
1538 from statistical analysis.
1539
1540 **18:** Figure 1.15 do statistical analysis.
1541 **Answer:** Thank you very much for this comment. I followed Xu Dongyang’s paper
1542 which did not do statistical analysis in qPCR data (Xu et al., 2017).
1543

The Concerns for Prof. Nakano:

1: Line 422, specify plant species that produce apocarotenoids.

Answer: Thank you very much for this comment. After revision, I add the example of species in apocarotenoid introduction (line 458-479)

2: Line 561-562 what does this mean.

Answer: Thank you very much for this comment. I revised introduction which matches to my study better. “Carotenoid is essential for photosynthesis. Photosynthesis is the primary pathway for plants to obtain energy. The inhibition of carotenoid biosynthesis enzyme PDS by the herbicide fluridone kills plants under natural conditions. However, under in vitro conditions with sucrose application, fluridone bleaches leaf pigments but does not kill plants, allowing them to develop flowers. In this condition, carotenoid deficiency leads to a reduction of PR growth. This suggests carotenoids play a role in root growth regulation. However, the detailed mechanism is not reported yet. In this study, I identify that carotenoid, as a source of apocarotenoids, play a role in the regulation of root growth mediated by endogenous auxin levels.” (line 626-634).

3: Line 702-704, chlorosis and root growth suppression which comes first.

Answer: Thank you very much for this comment. As stated in intermediate defense, after fluridone treatment, leaf bleaching and chlorosis occurs 2 or 3 days, which is slower than root growth suppression, although I have not done time course of carotenoid quantification. It can exclude the root growth suppression by fluridone from secondary pleiotropic effect.

4: Line 724, why *psy* mutant responds to fluridone.

Answer: Thank you very much for this comment. One possibility is that *psy* mutant used in this study is not a null mutant of the *PSY* gene.

1573

1574 **5:** Line 730 what happens between 36 and 48 h after fluridone treatment.

1575 **Answer:** Thank you very much for this comment. We can see in this time period the
1576 root growth of WT in both mock and fluridone treatment was reduced slightly.
1577 Since it is a time-lapse imaging, actually I do not know what happens
1578 between this time period. What I can imagine is that maybe there is something
1579 disturbing root growth. But I think it does not affect the results we can get
1580 from this graph.

1581

1582 **6:** Line 757, *DR5:LUC* expression induction at the initial transfer.

1583 **Answer:** Thank you very much for this comment. I think it results from the transfer
1584 stress, and it occurs both in WT and *mPDS* plants, and in mock and fluridone
1585 treatment.

1586

1587 **7:** Line 763, what happens at 108 hat.

1588 **Answer:** Thank you very much for this comment. I think in this time period long-term
1589 fluridone treatment starts to reduce auxin signaling. Although it is not
1590 observed in this study, I predict that in this time period the effect of carotenoid
1591 deficiency caused by fluridone becomes large to affect the status which is
1592 necessary for plant growth. Thus plant development shows overall decline.

1593

1594 **8:** Line 766 time course of carotenoid quantification.

1595 **Answer:** Thank you very much for this comment. I am sorry that I have not done time
1596 course of carotenoid quantification by fluridone. The statement here is a
1597 prediction, since long-term fluridone incubation results in the bleaching of
1598 the whole plants, thus severe low carotenoid levels can be predicted.

1599

1600 **9:** Line 787, quick introduction of *wei2 wei7* mutants

1601 **Answer:** Thank you very much for this comment. After revision, I add an introduction

of WEI2 WEI7 “WEI2 and WEI7 encode the subunits of anthranilate synthase, which is responsible for the synthesis of tryptophan precursor, anthranilate (Stepanova et al., 2005).” (line 855-857)

10: Line 793-794 the discussion of root growth encouragement by fluridone in *yucQ* mutant.

Answer: Thank you very much for this comment. The induction of auxin levels in *yucQ* by fluridone is unknown yet. In this study the possibility that the inhibition of auxin catabolism is raised. But there are also other possible explanations, such as IPyA-independent auxin biosynthesis pathway.

11: the difference between *yucQ* mutant and YDF treatment with fluridone treatment.

Answer: Thank you very much for this comment. The related discussion is in the manuscript “One possibility is that auxin biosynthesis in *yucQ* is not completely abolished, allowing fluridone to induce auxin within suboptimal levels, consequently inducing root growth. However, a high dose of YDF completely blocks root local auxin biosynthesis from the IPyA pathway, thus fluridone cannot elevate endogenous auxin levels to suppress root growth. To address it, the endogenous IAA levels of root tissues in *yucQ* and YDF-treated plants after fluridone treatment are necessary in the future. Another hypothesis is that fluridone inhibits enzymes involved in the IG biosynthesis pathway, such as SUR1 and SUR2. In this hypothesis, fluridone reduces SUR1 or SUR2 activity to overflow auxin biosynthesis from the IPyA pathway in *yucQ* plants; however, this overflow is abolished by YDF which inhibits YUCCA activities.” (line 995-1005)

12: Line 891-894, how retinal controls auxin contents and response.

Answer: Thank you very much for this comment. In this study the possibility that the inhibition of auxin catabolism is raised. But there are also other possible

explanations, such as retinal directly reduces auxin signaling, or targets IPyA-independent pathway. Also it is necessary to measure endogenous IAA levels following co-treatment with NAA and retinal, as well as KKI and retinal. the related discussion is in the manuscript. (line 1076-1085)

13: Line 1010, confirmation of retinal changes auxin levels.

Answer: Thank you very much for this comment. As stated in intermediate defense, up to now I have no data to assume the modulation of auxin levels by retinal. The quantification of IAA levels by retinal in short time course is necessary in the future (line 1069-1071).

14: Chapter 2 comments.

Answer: Thank you very much for the comments in Chapter 2. After revision, I rearranged the contents in Chapter 2. Thus the comments for Chapter 2 are mainly abolished. For line 1236 “*pkl* mutant data”, I deleted the figure of LR distribution.

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Tables

Table 1.1 Gene-specific primers used for qRT–PCR analysis.

Gene name	Primer name	Primer Sequence (5'-3')	Reference
<i>ACTIN2</i>	ACTIN2-27S	5'-CGCTCTTTCTTTCCAAGCTCATA-3'	Xu et al., 2017
	ACTIN2+55 AS	5'-CCATACCGGTACCATTGTCACA-3'	
<i>IAA19</i>	IAA19+390S	5'-CTTCGGTTTCCGTGGCATCG-3'	Xu et al., 2017
	IAA19+521 AS	5'-CATGACTCTAGAAACATCCC-3'	
<i>YUC9</i>	YUC9+542S	5'-ATAAGTCCGGCGAGAAATTCAGAG-3'	Xu et al., 2017
	YUC9+682 AS	5'-TCGGTAAAACATGAACCGAG-3'	
<i>CCR1</i>	CCR1+3599 S	5'-AAGAGCAAGCGTTTGACAGGTAAG-3'	This study
	CCR1+3678 AS	5'-TACGATTGCGATGAAGGAAGTGG-3'	
<i>CCR2</i>	CCR2+2888 S	5'-CATCGGGTAGCTGCTGATATTGGG-3'	This study
	CCR2+2968 AS	5'-AGCCAACCAAGTAAACCAAGAAGG-3'	
<i>CCD1</i>	CCD1+2574 S	5'-TATGTTCCGCGTGAGACAGCAG-3'	This study
	CCD1+2665 AS	5'-TCACAGTCACGCATGATTTC-3'	
<i>CCD7</i>	CCD7+2077 S	5'-CGTTGGTGAGCCCATGTTTGTC-3'	This study
	CCD7+2180 AS	5'-TCTCTCCACCGAAACCGCATACTC-3'	
<i>CCD8</i>	CCD8+2715 S	5'-GTGCAACCCATGAGGATGATGGAG-3'	This study
	CCD8+2839 AS	5'-CCATAGGGAACTTGGCTCTTGC-3'	

Figures

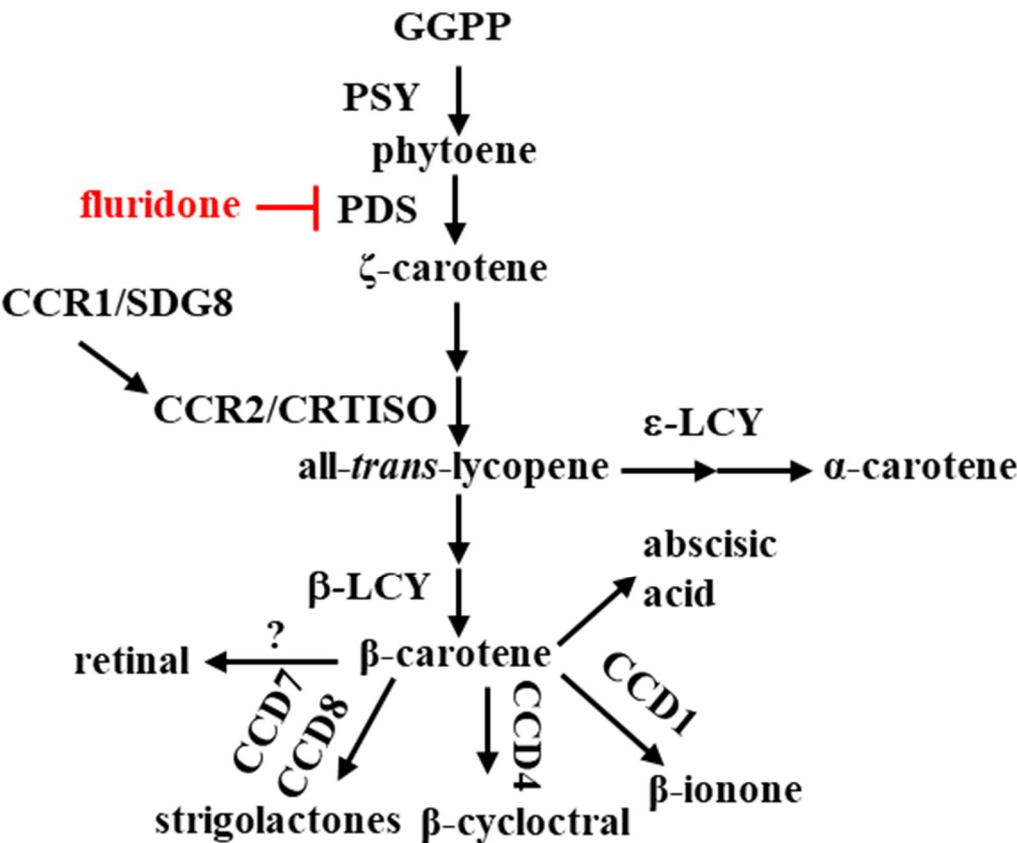
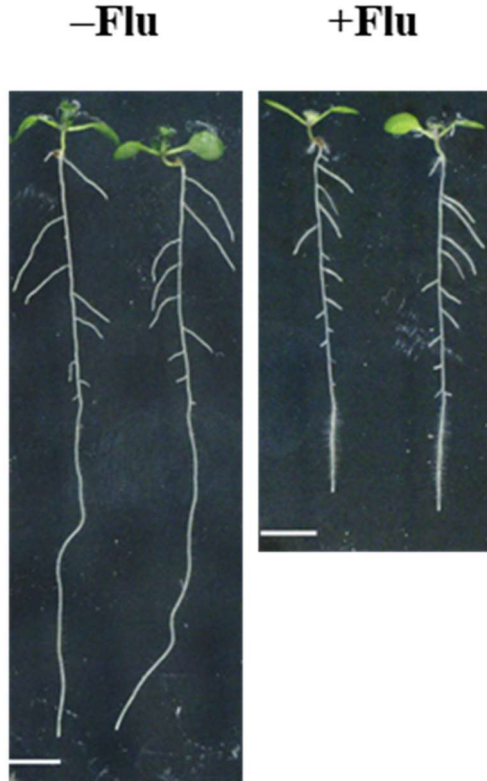


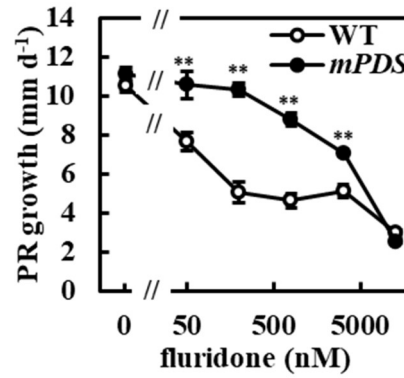
Figure 1.1 The carotenoid metabolism pathway.

PSY, phytoene synthase; PDS, phytoene desaturase; CCR1/SDG8, carotenoid chloroplast regulatory1/set domain group 8; CCR2/CRTISO, carotenoid chloroplast regulatory2/crtiso; β-LCY, β-cyclase; ε-LCY, ε-cyclase; CCD1/4/7/8, carotenoid cleavage dioxygenase 1/4/7/8.

A



B



C

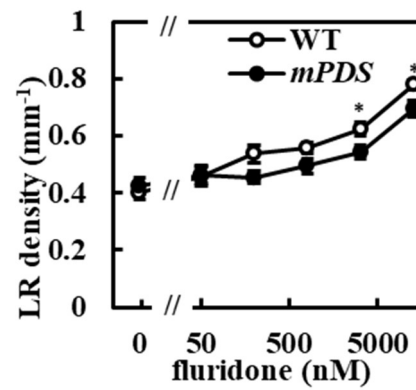


Figure 1.2 Fluridone inhibits phytoene desaturase activity to suppress PR growth and induce LR density in a dose-dependent manner.

Five-day-old plants of WT and *mPDS* were transferred to medium with or without fluridone. (A) Photographs of WT in mock and 12.8 μ M fluridone medium were captured at 3 dat. Scale bar = 5 mm. The PR growth rate (B) was analyzed from 2 dat to 3 dat. The LR density (C) was counted at 2 dat. Data represent the means $\pm$ standard error (SE) from 12 seedlings. *Significant differences compared to WT (*: *P* < 0.05, **: *P* < 0.01; Student's *t*-test).

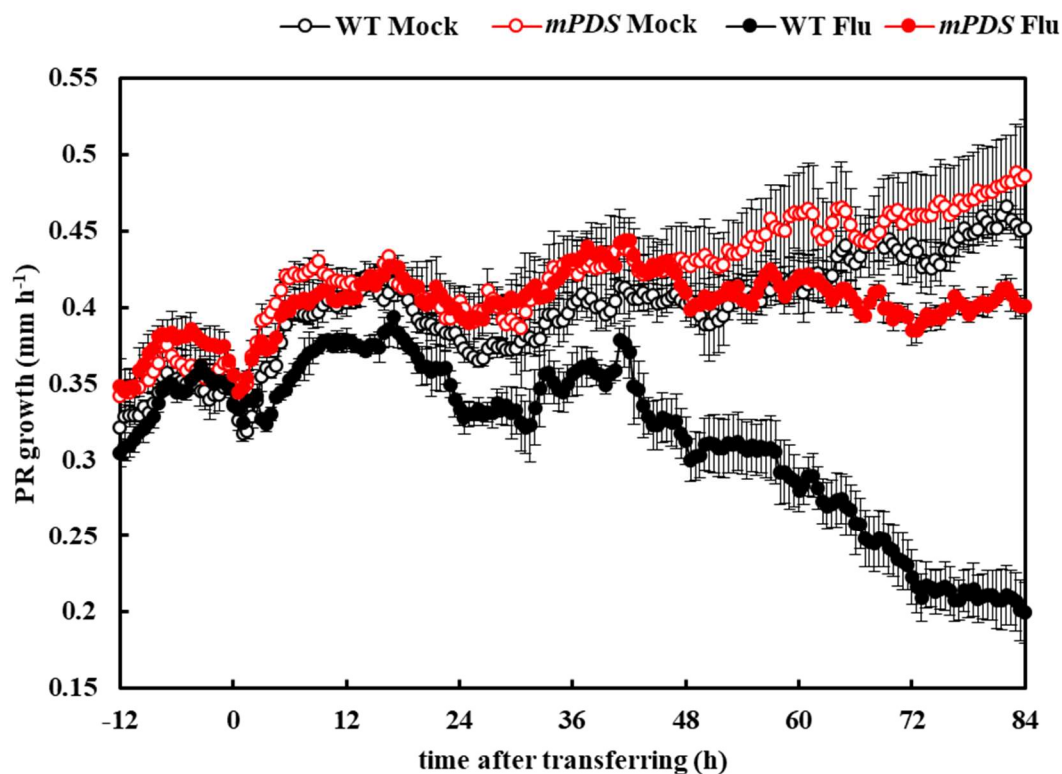


Figure 1.3 The kinetic growth rate of PRs in WT and *mPDS* after fluridone treatment.

Four-day-old plants of WT and *mPDS* were transferred to mock medium for a 1 d preincubation. Then plants were transferred to new medium with or without 800 nM fluridone at time 0. The PR growth rate was measured from preincubation at an interval of 30 min, which was indicated as WT mock-treated plants (hollow black), WT fluridone-treated plants (solid black), *mPDS* mock-treated plants (hollow red), and *mPDS* fluridone-treated plants (solid red). Data represent the means $\pm$ SE from 5 seedlings.

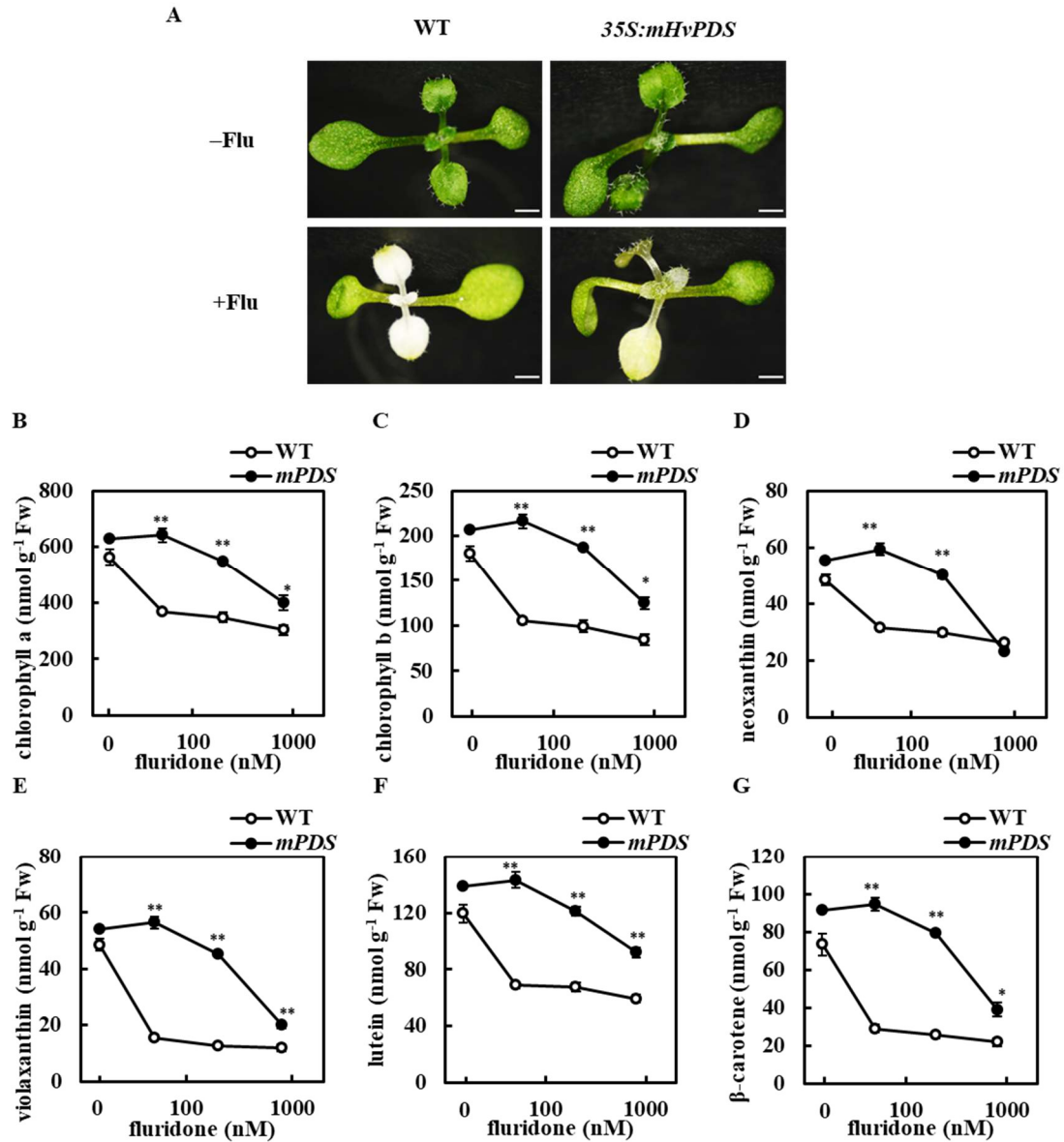


Figure 1.4 Fluridone reduces chlorophyll and carotenoid contents, and *mPDS* plants are resistant to it.

(A–G) Five-day-old plants of WT and *mPDS* were transferred to medium with or without 800 nM fluridone. (A) Leaf pigment bleaching effect of WT and *mPDS* by fluridone. Photographs were captured at 3 dat. Scale bar = 5 mm. Chlorophyll a (B), chlorophyll b (C), neoxanthin (D), violaxanthin (E), lutein (F) and β-carotene (G) contents in the whole plants of WT (hollow circle) and *mPDS* (solid circle) were quantified at 2 dat. Data represent the means ± SE from 3 biological replicates.

Significant differences compared to WT (: $P < 0.05$, **: $P < 0.01$; Student's t -test).

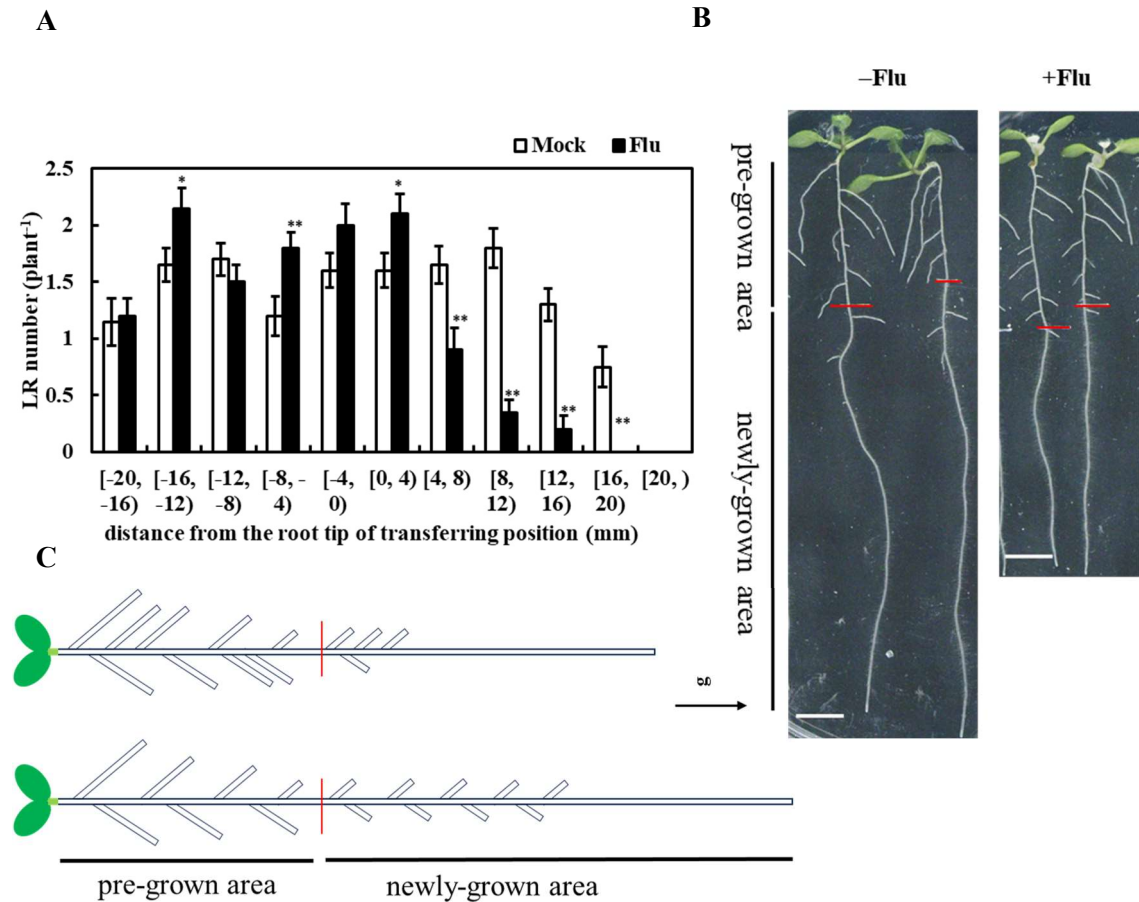


Figure 1.5 Fluridone induces LR formation in the pre-grown areas of PR but severely reduces it in the newly grown areas of PR.

(A, B) Five-day-old plants were transferred to new medium with or without 800 nM fluridone. The number of LRs was counted at 4 dat. The root tips of the transferring positions were positioned as 0. The pre-grown root was positioned as < 0 . The newly grown root was positioned as > 0 . Photographs were captured at 4 dat. Scale bar = 5 mm. (C) Schematic showing the positioning of PR and LR emergence in mock (below) and fluridone treatment (upper) at 4 dat. Red lines indicate the root tips of the transferring position. Data represent the means $\pm$ SE from 20 seedlings. *Significant differences compared to mock-treated plants (*: $P < 0.05$, **: $P < 0.01$; Student's t -test).

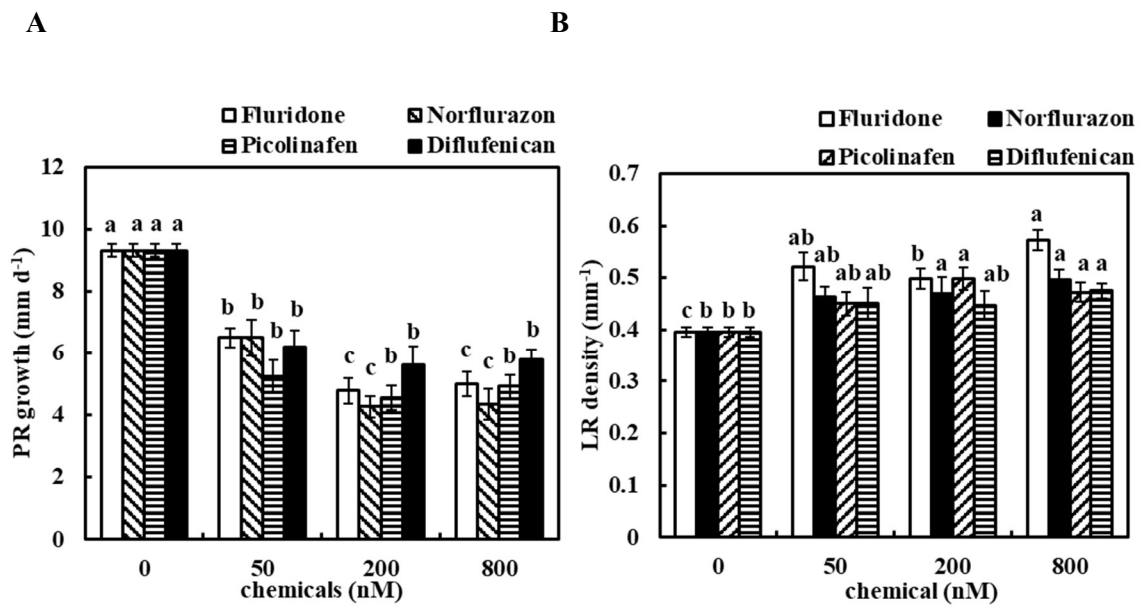


Figure 1.6 PDS inhibitors exhibit a fluridone-like effect on PR suppression and LR induction.

(A, B) Five-day-old plants were transferred to new medium with or without PDS inhibitors. The PR growth rate (A) was analyzed from 2 to 3 dat. The LR density (B) was counted at 3 dat. The bars indicate fluridone (white), norflurazon (diagonal striped black), picolinafen (horizontal striped black), and diflufenican (black). Data represent the means $\pm$ SE from 8 to 14 seedlings. Different lowercase letters above the bars indicate significant differences at $P < 0.05$ (one-way ANOVA following Tukey-Kramer test).

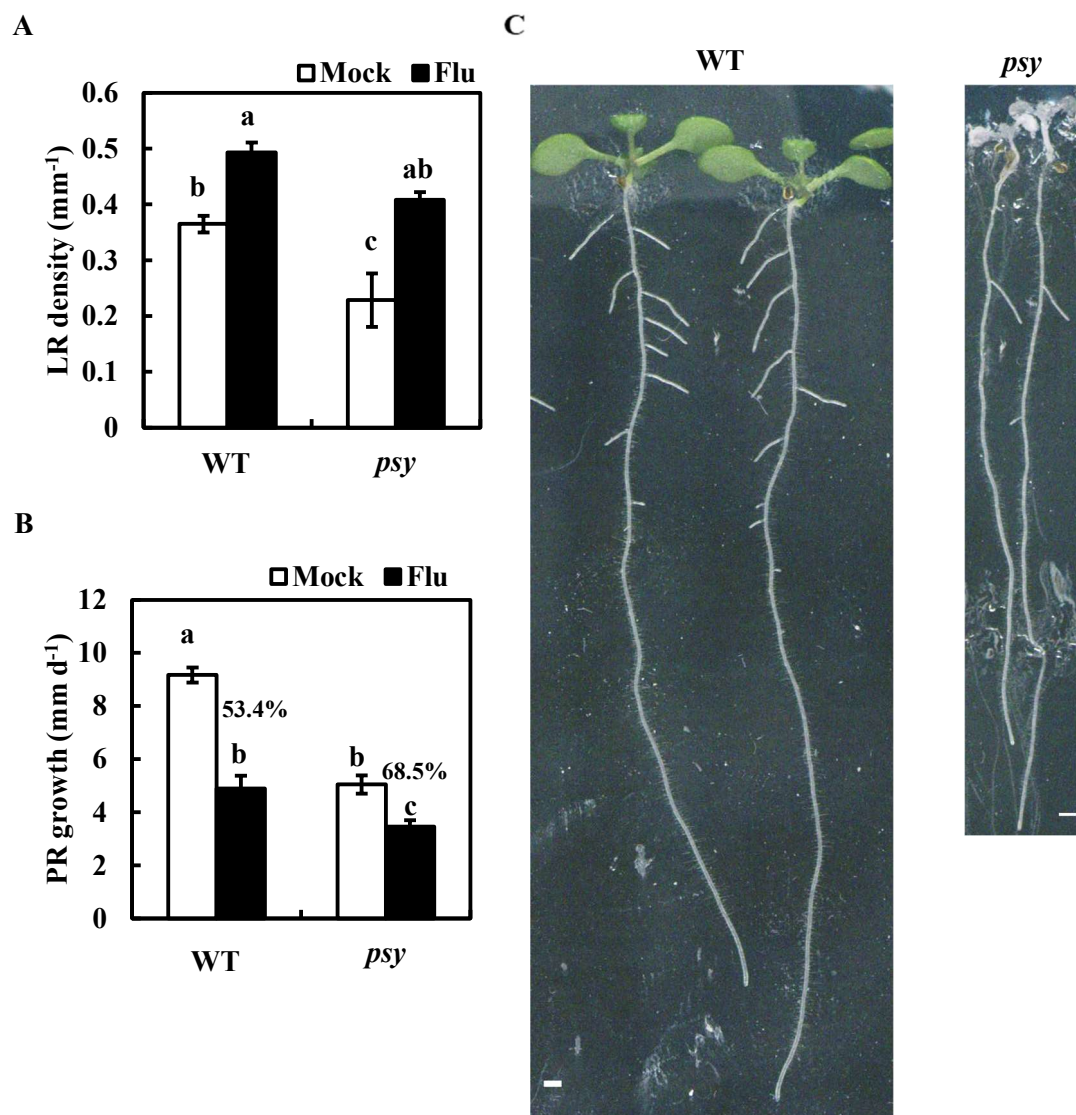


Figure 1.7 The phenotypes and fluridone resistance of the *psy* mutant.

(A, B) Five-day-old plants of WT and *psy* were transferred to medium with or without 800 nM fluridone. The PR growth rate (A) was analyzed from 2 to 3 dat. The LR density (B) was counted at 3 dat. (C) The pictures of WT and *psy* mutant captured at 8 d after germination (without transfer). Scale bar = 1 mm. The percentage in (B) means the relative PR growth rate in fluridone-treated plants compared to mock-treated plants. Data represent the means $\pm$ SE from 10 seedlings in (A, B). Different lowercase letters above the bars indicate significant differences at $P < 0.05$ (one-way ANOVA following Tukey-Kramer test).

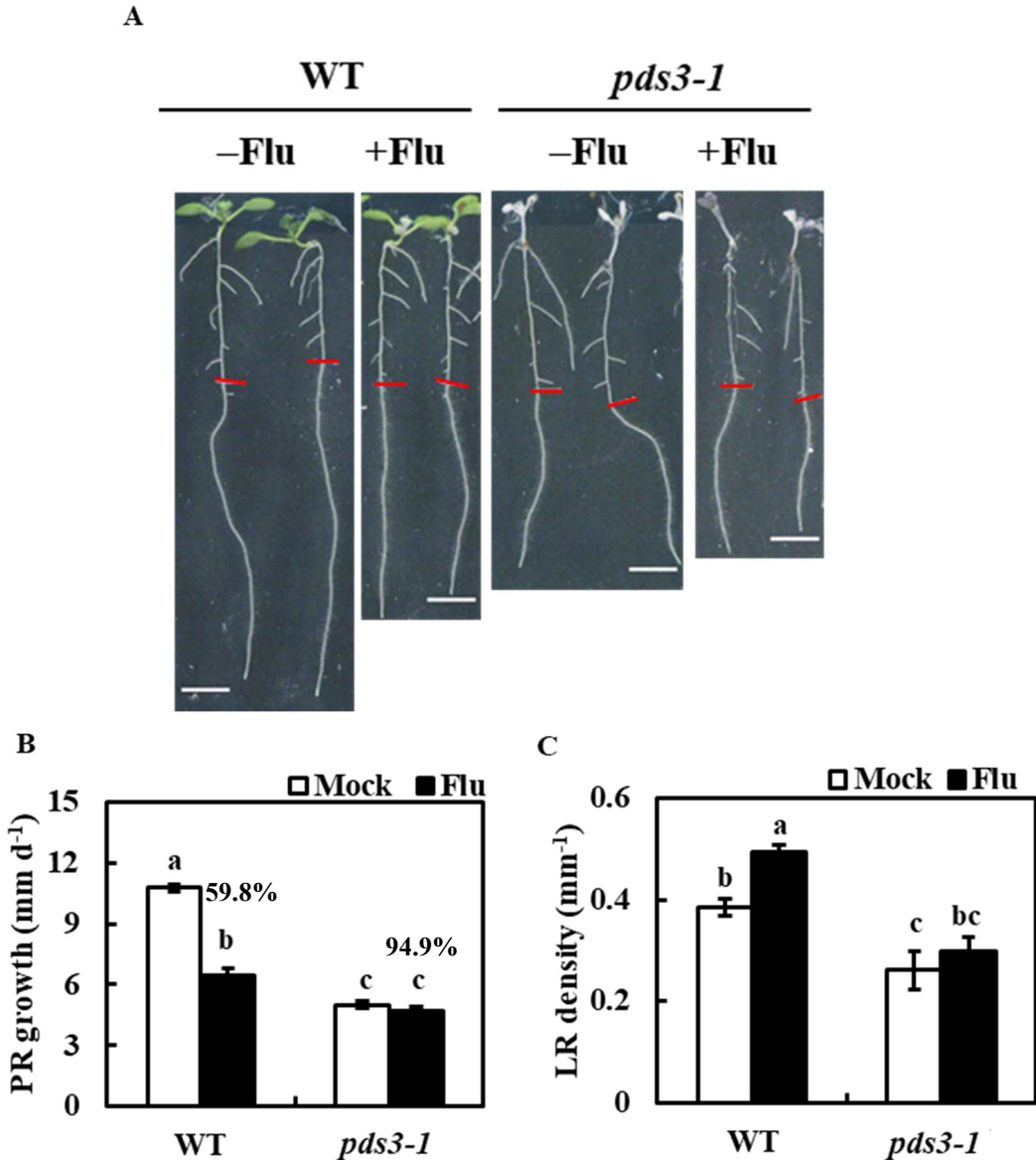


Figure 1.8 The change of root architecture by fluridone is specifically dependent on the activity of PDS.

Five-day-old plants of WT and *pds3-1* were transferred to medium with or without 800 nM fluridone. Photographs were captured at 3 dat. Scale bar = 5 mm. Red lines indicate the root tips at the time point of transfer. The PR growth rate in (B) was analyzed from 2 to 3 dat. The LR density in (C) was counted at 3 dat. The percentage in (B) means the relative PR growth rate in fluridone-treated plants compared to mock-treated plants. Data represent the means $\pm$ standard error (SE) from 10 seedlings. Different lowercase letters above the bars indicate significant differences at $P < 0.05$ (one-way Analysis of Variance [ANOVA] following Tukey-Kramer test).

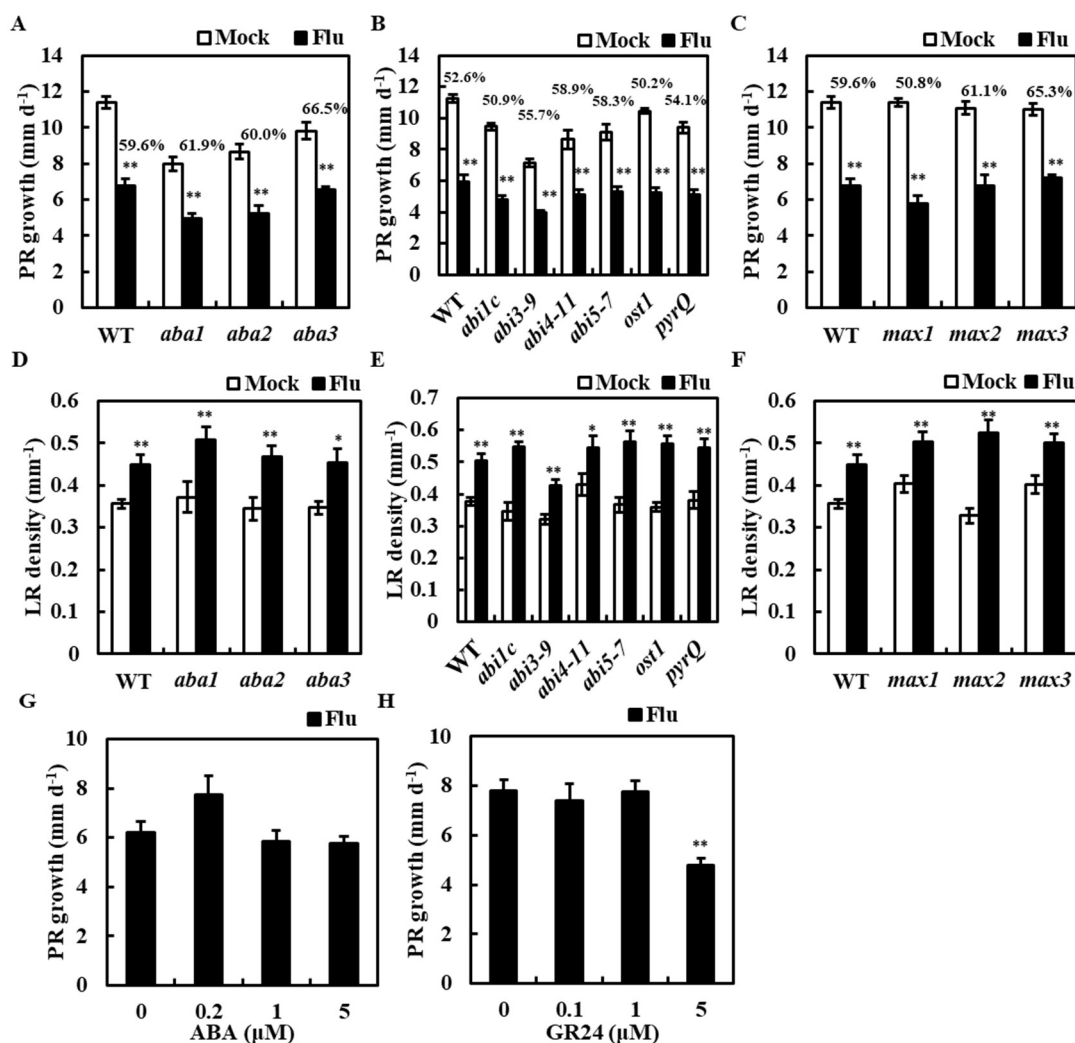


Figure 1.9 Fluridone mediates root architecture via ABA- and SL-independent manner.

(A–F) The fluridone resistance in ABA- and SL-related mutants. Five-day-old plants were transferred to medium with or without 800 nM fluridone. (A, D) ABA biosynthesis mutants. (B, E) ABA signaling mutants. (C, F) SL biosynthesis and signaling mutants. (G, H) Five-day-old plants were transferred to medium with or without fluridone and ABA (G) or *rac*-GR24 (H). The PR growth rate was analyzed from 2 to 3 dat. The LR density was counted at 3 dat. The percentage in (A–C) means the relative PR growth rate in fluridone-treated plants compared to mock-treated plants. Data represent the means $\pm$ SE from 10 seedlings. *Significant differences compared to mock-treated plants in (A–F) or non SL-treated plants in (H) (*: $P < 0.05$, **: $P < 0.01$; Student's *t*-test). n.s. indicates no significant difference.

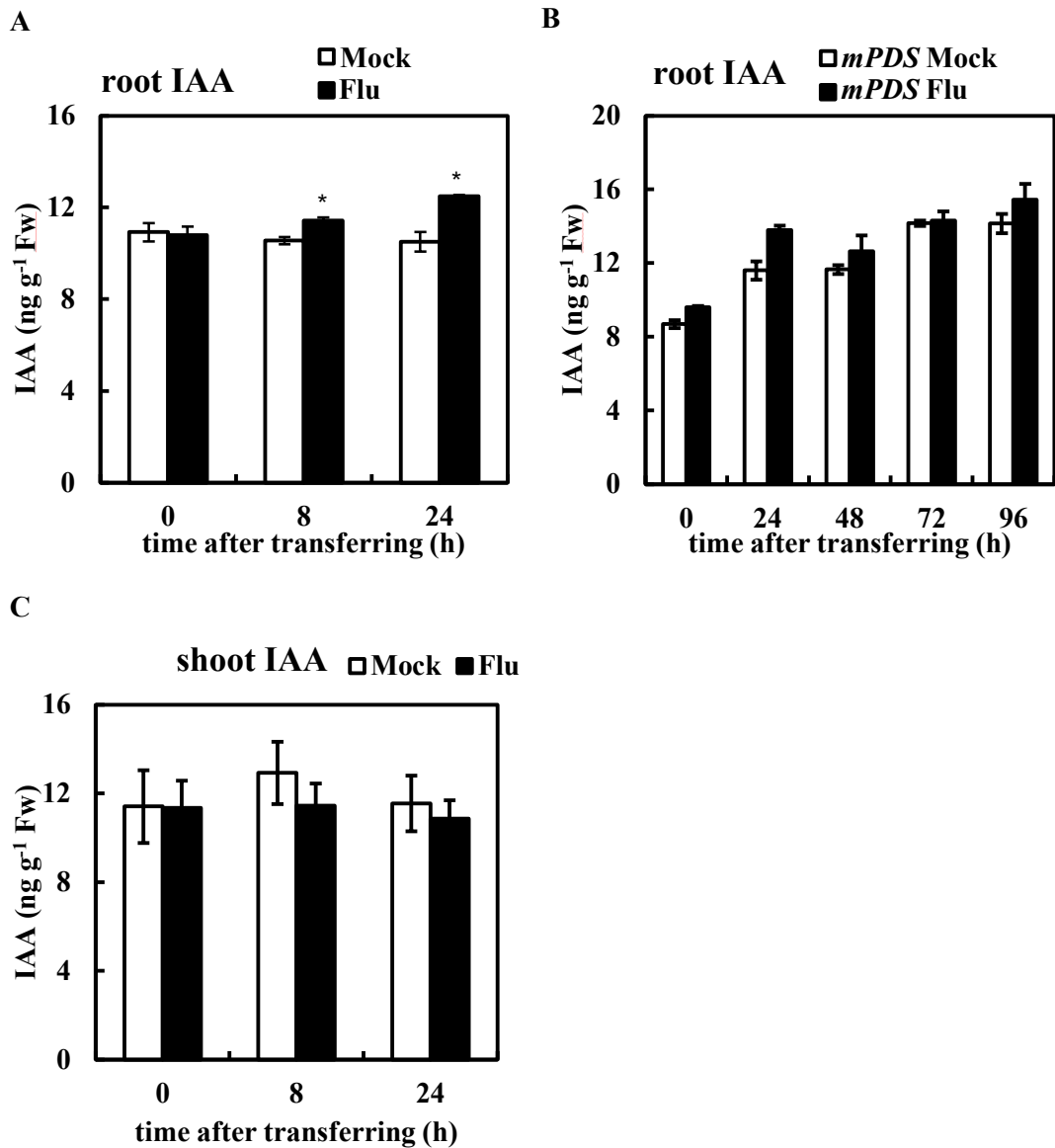
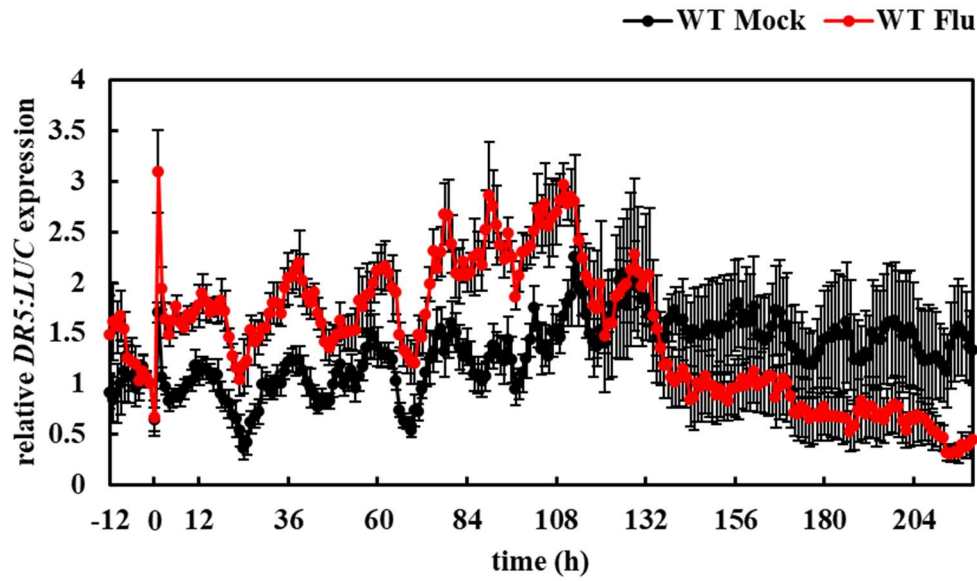


Figure 1.10 Fluridone induces endogenous IAA levels in the roots of WT plants but not in the shoots.

(A–C) Five-day-old plants were transferred to new medium with or without 800 nM fluridone at time 0. The IAA contents in the roots of WT (A) and *mPDS* (B) and in the shoots of WT (C) were measured at the indicated time points. Data represent the means $\pm$ SE from 3 biological replicates. *Significant differences compared to mock plants (*: $P < 0.05$; Student's *t*-test).

A



B

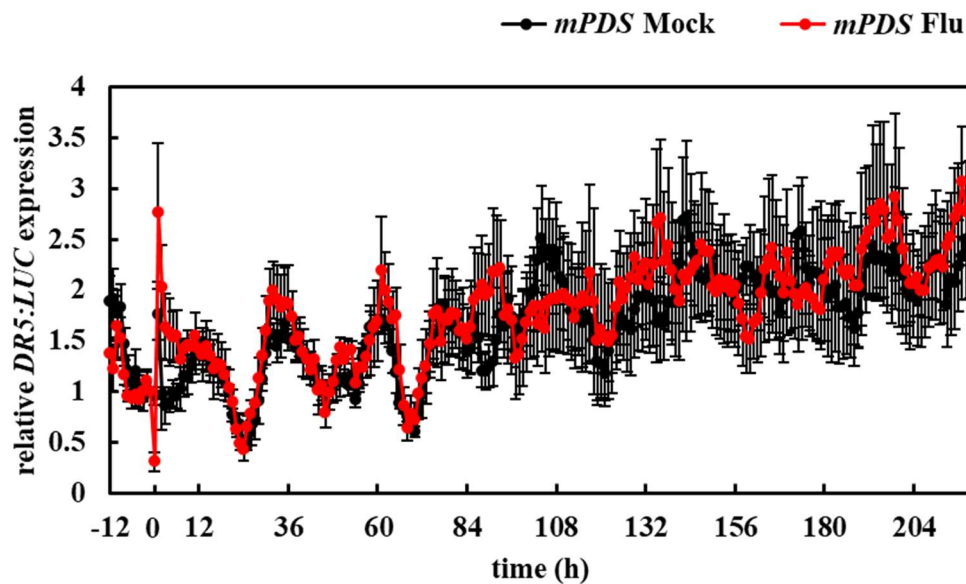


Figure 1.11 The expression of *DR5:LUC* at the root tips was increased following fluridone treatment.

Four-day-old plants grown on 100 μ M D-luciferin medium were transferred to D-luciferin-containing medium for a 1 d preincubation. Then plants were transferred to new D-luciferin-containing medium with or without 800 nM fluridone at time 0. The *DR5:LUC* expression in WT (A) and *mPDS* (B) was measured. Data represent the means (relative to the mean of time -1 h) $\pm$ SE from 6 seedlings.

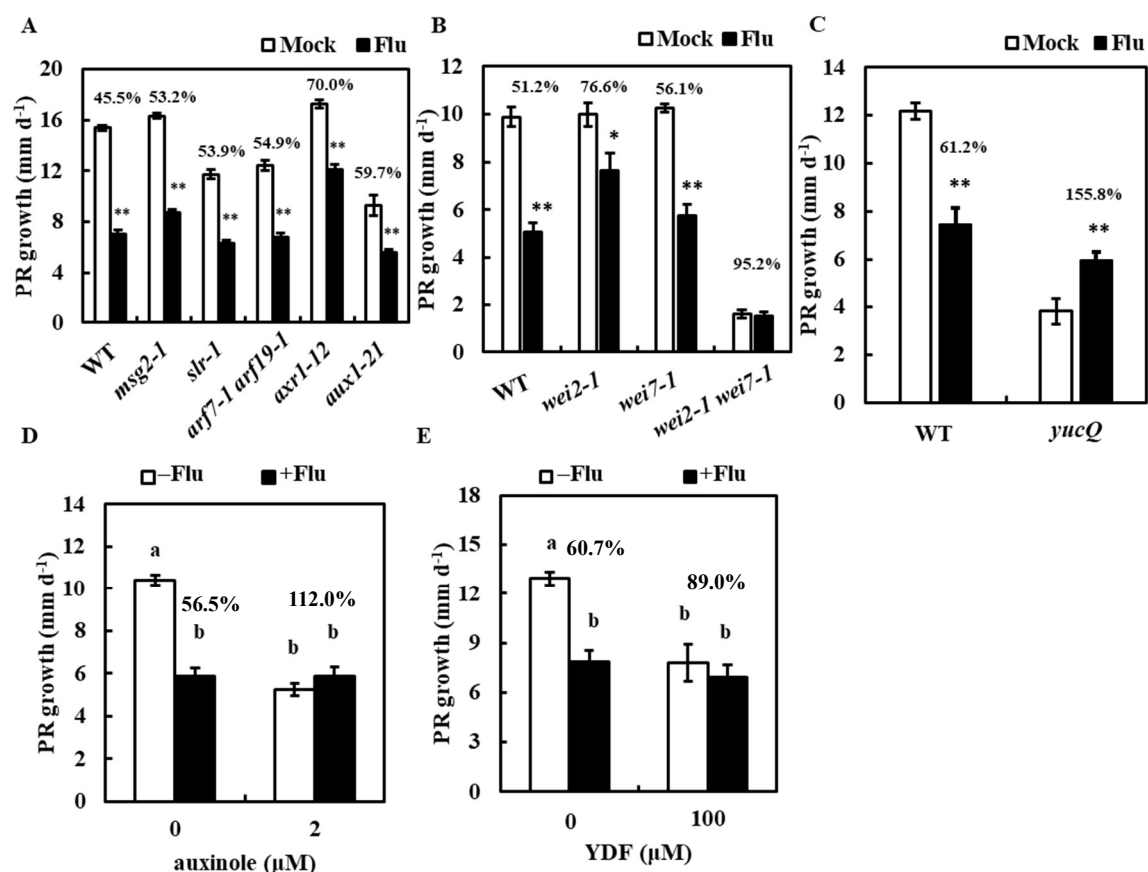


Figure 1.12 Auxin biosynthesis, signaling, and influx transport are required for fluridone-mediated root growth regulation.

(A–C) The resistance to fluridone in auxin-related mutants. Five-day-old plants were transferred to new medium with or without 800 nM fluridone. (A) auxin signaling and influx transport mutants. (B) *wei2-1*, *wei7-1* and *wei2-1 wei7-1* mutants. (C) *yucQ* mutant. (D, E) Five-day-old plants were transferred to new medium with or without 800 nM fluridone and 2 μM auxinole (D) or 100 μM YDF (E). The PR growth rate was analyzed from 2 to 3 dat. The percentage in (A–E) means the relative PR growth rate in fluridone-treated plants compared to mock-treated plants. Data represent the means ± SE from 20 seedlings in (A) and from 8 seedlings in (B–E). Different lowercase letters above the bars in (D, E) indicate significant differences at $P < 0.05$ (one-way ANOVA following Tukey-Kramer test). *Significant differences compared to mock-treated plants in (A–C) (*: $P < 0.05$, **: $P < 0.01$; Student's t -test).

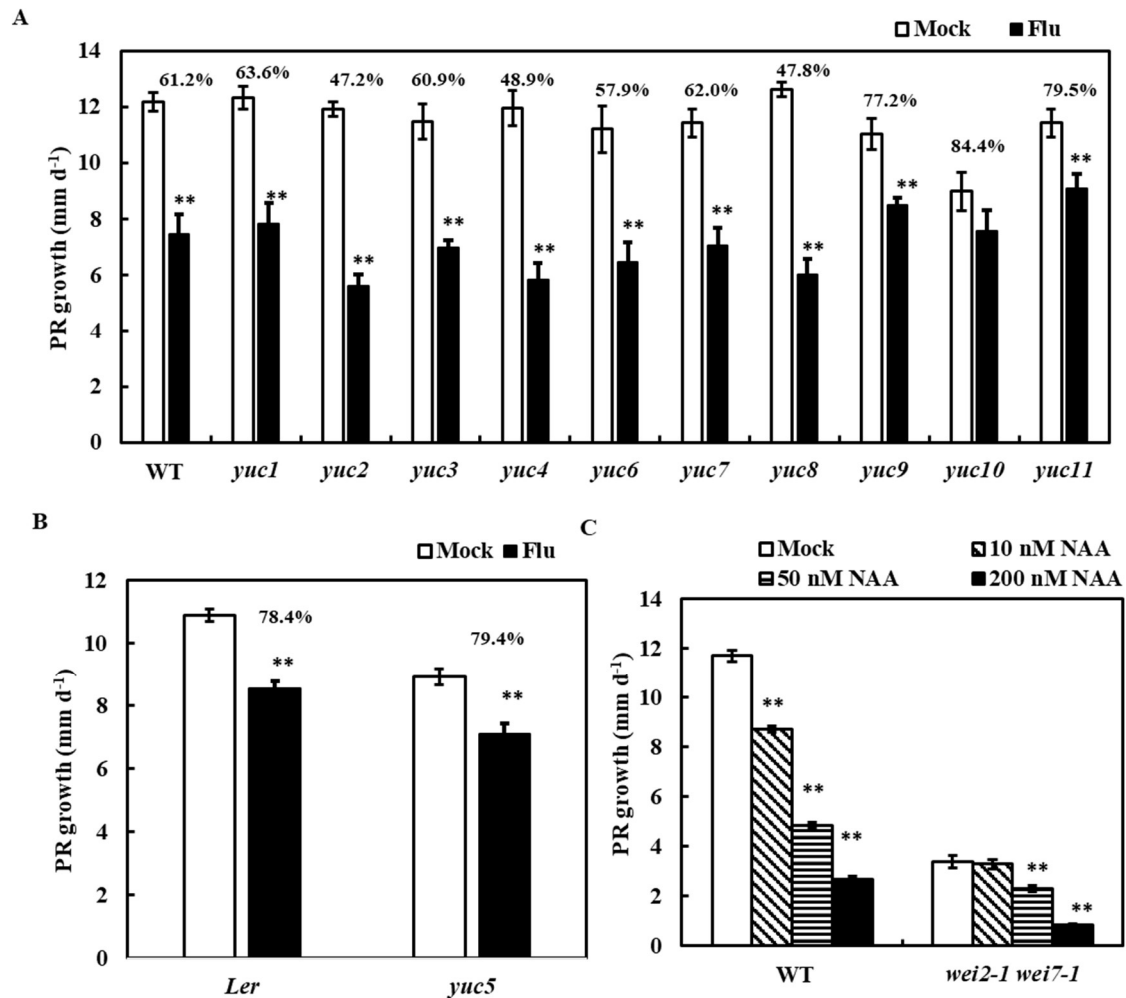


Figure 1.13 The *yuc* single mutants have weak or no resistance to fluridone, and *wei2-1 wei7-1* shows resistance to low dose of exogenous NAA. (A, B) Five-day-old plants were transferred to medium with or without 800 nM fluridone. (A) The *yuc* mutants in a Col-0 background. (B) The *yuc* mutant in a Landsberg *erecta* background. (C) Five-day-old plants of WT and *wei2-1 wei7-1* were transferred to medium with or without NAA. The PR growth rate was analyzed from 2 to 3 dat. The percentage in (A, B) means the relative PR growth rate in fluridone-treated plants compared to mock-treated plants. The bars in (B) indicate mock (white), 10 nM NAA (diagonal stripe black), 50 nM NAA (horizontal striped black) and 200 nM NAA (black). Data represent the means $\pm$ SE from 8-10 seedlings. *Significant differences compared to mock-treated plants (**: $P < 0.01$; Student's *t*-test).

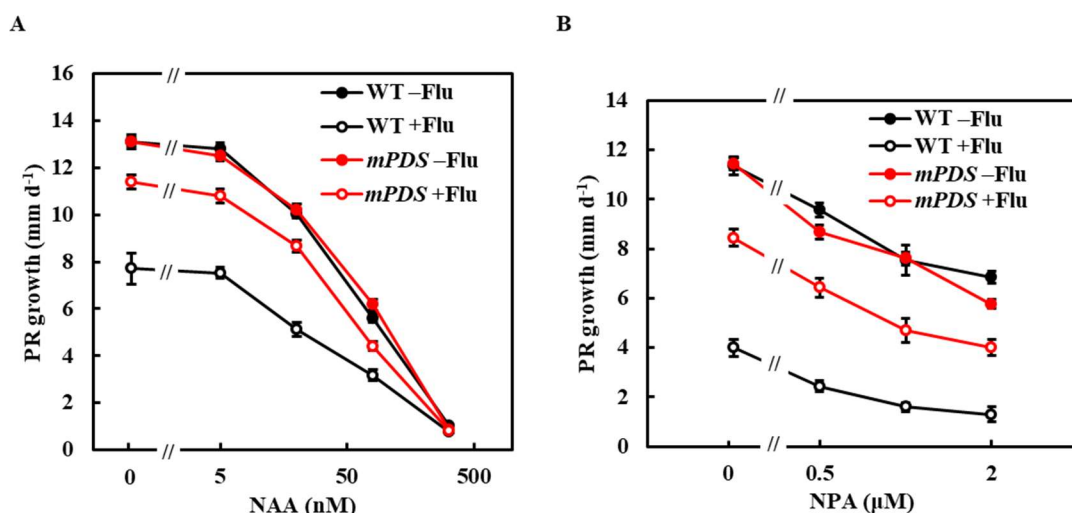


Figure 1.14 Exogenous auxin and NPA suppress PR growth in an additive manner with fluridone.

(A, B) Five-day-old plants of WT and *mPDS* were transferred to medium with or without 800 nM fluridone and NAA (A) or NPA (B). The PR growth rate was analyzed from 2 to 3 dat in (A) or 3 to 4 dat in (B). Data represent the means $\pm$ SE from 8 seedlings. For (A), the relative PR growth of fluridone-treated plants to non-fluridone-treated plants is as follows: 58.8% (WT, 0 nM NAA), 86.8% (*mPDS*, 0 nM NAA), 58.9% (WT, 5 nM NAA), 86.3% (*mPDS*, 5 nM NAA), 51.0% (WT, 20 nM NAA), 84.7% (*mPDS*, 20 nM NAA), 47.2% (WT, 80 nM NAA), 79.9% (*mPDS*, 80 nM NAA), 72.9% (WT, 320 nM NAA) and 97.8% (*mPDS*, 320 nM NAA). For (B), the relative PR growth of fluridone-treated plants to non-fluridone-treated plants is as follows: 35.1% (WT, 0 μ M NPA), 73.8% (*mPDS*, 0 μ M NPA), 25.5% (WT, 0.5 μ M NPA), 73.9% (*mPDS*, 0.5 μ M NPA), 21.2% (WT, 1 μ M NPA), 61.9% (*mPDS*, 1 μ M NPA), 19.0% (WT, 2 μ M NPA) and 69.8% (*mPDS*, 2 μ M NPA).

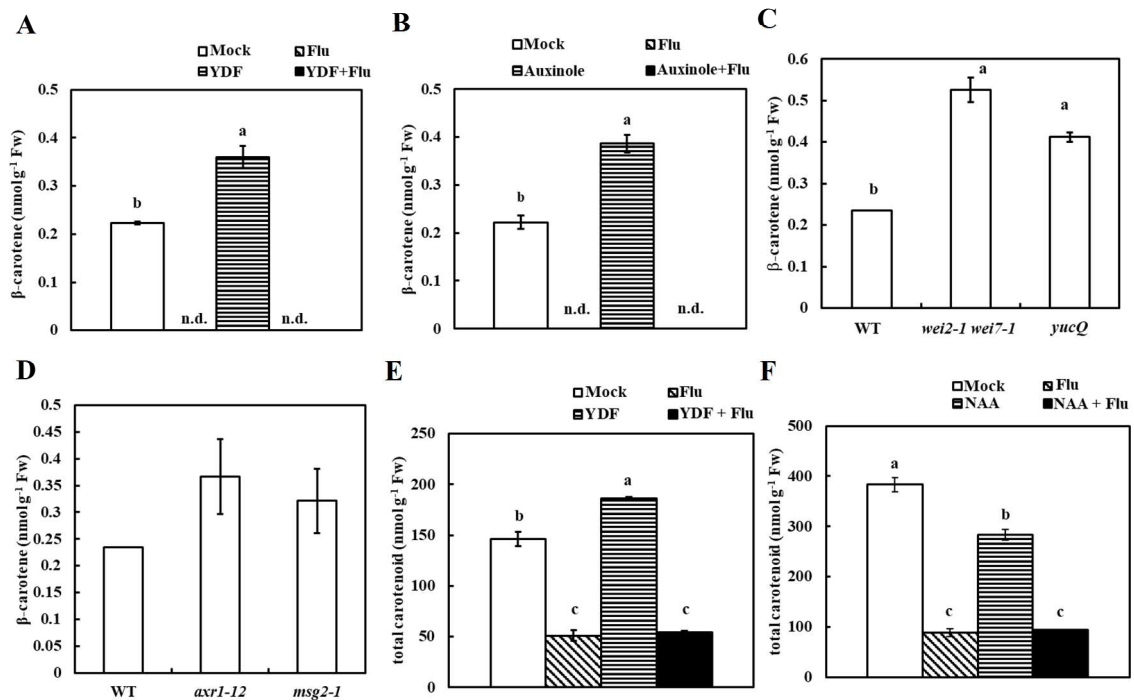


Figure 1.15 The regulation of carotenoid contents by auxin in roots and shoots.

(A–D) Five-day-old plants were transferred to medium with or without 800 nM fluridone and 100 μ M YDF (A, C), 10 μ M auxinole (B) or 320 nM NAA (D). The levels of β -carotene in the roots (A, B) were measured at 2 dat. The levels of total carotenoids in the shoots were measured at 4 dat in (C) and at 5 dat in (D). (E, F) The β -carotene levels of auxin-related mutants in the roots were measured at 7 d after germination. Data represent the means $\pm$ SE from 3 biological replicates. The bars indicate mock (white), 800 nM fluridone (diagonal striped black), YDF (A, C) or auxinole (B) or NAA (D) (horizontal striped black) and fluridone + YDF (A, C) or fluridone + auxinole (B) or fluridone + NAA (D) (black). Different lowercase letters above the bars in (A–E) indicate significant differences at $P < 0.05$ (one-way ANOVA following Tukey-Kramer test). n.d. indicates no detectable data.

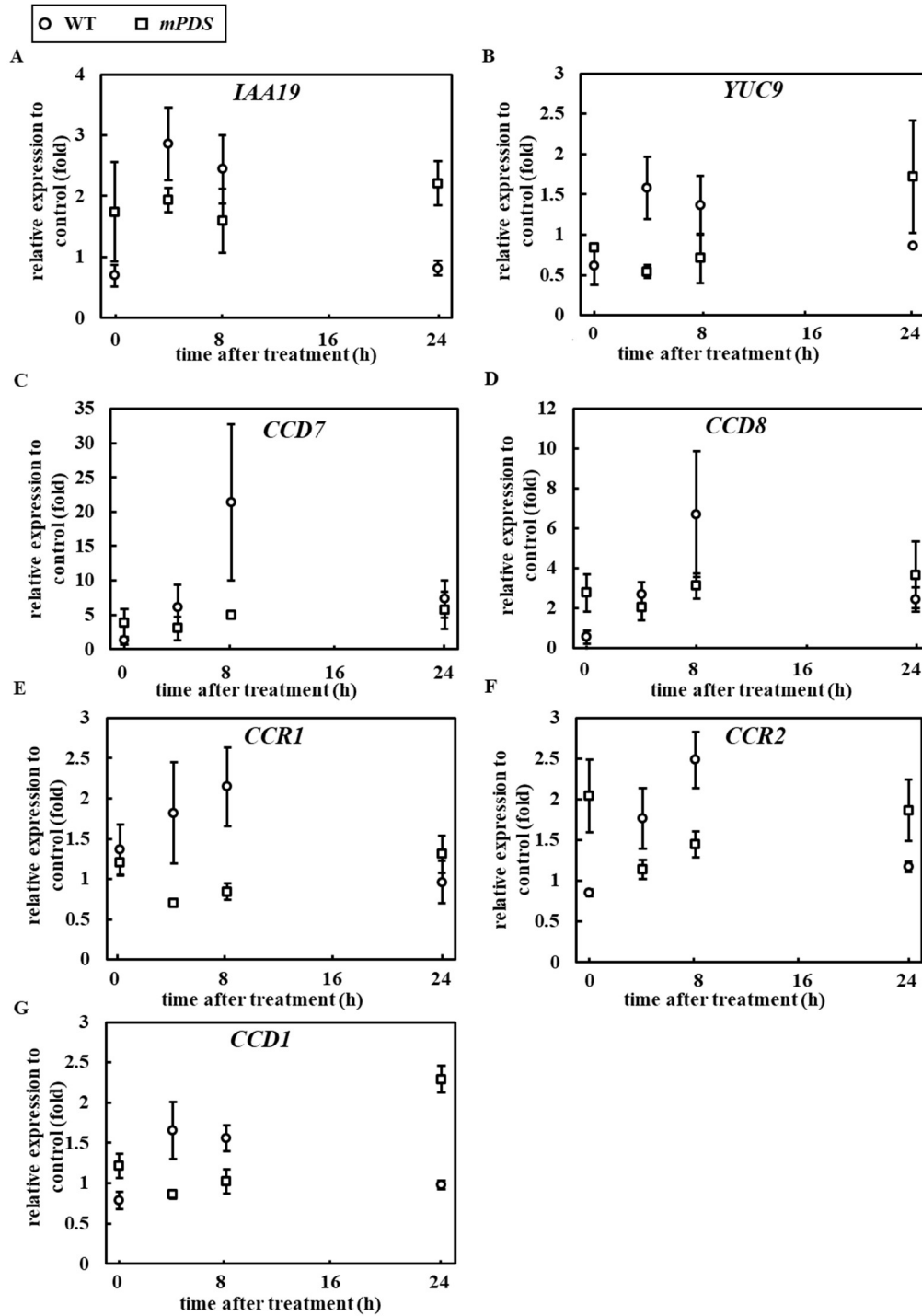
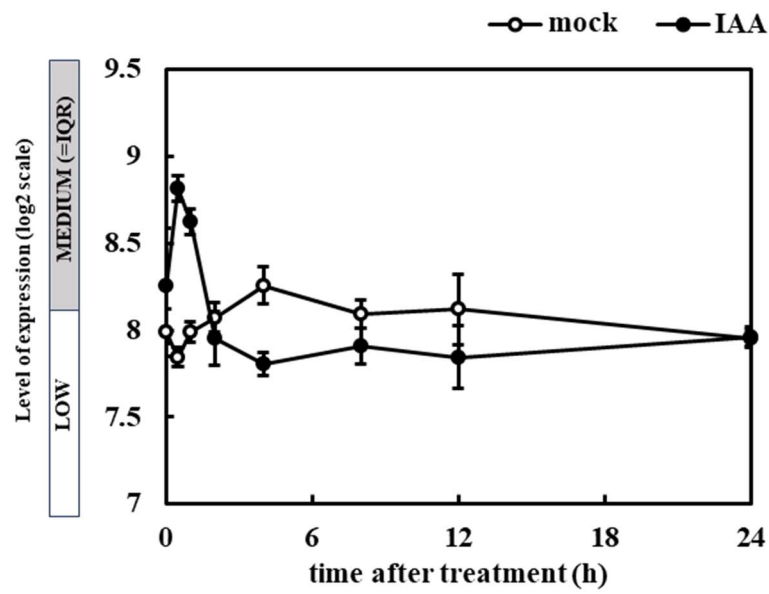


Figure 1.16 The expression of auxin biosynthesis, response, carotenoid biosynthesis and catabolism genes by fluridone treatment.

Five-day-old plants of WT and *mPDS* were transferred to medium with or without 800 nM fluridone at time 0. (A–G) Relative expression of *IAA19*, *YUC9*, *CCD7*, *CCD8*, *CCR1*, *CCR2*, and *CCD1* after fluridone treatment in the WT (open circle) and *mPDS* (open square). Data represent the means $\pm$ SE from 3 biological replicates.

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2548

2549 Figure 1.17 *CCD7* expression is rapidly induced by exogenous IAA treatment.

2550 Public microarray data were retrieved and analyzed using GENEVESTIGATOR and

2551 the expression of *CCD7* was plotted based on Affymetrix Arabidopsis ATH1 Genome

2552 Array data (Experiment ID: AT-00655). Data are expressed as log2 scale and represent

2553 the means $\pm$ SE from 3 biological replicates.

2554

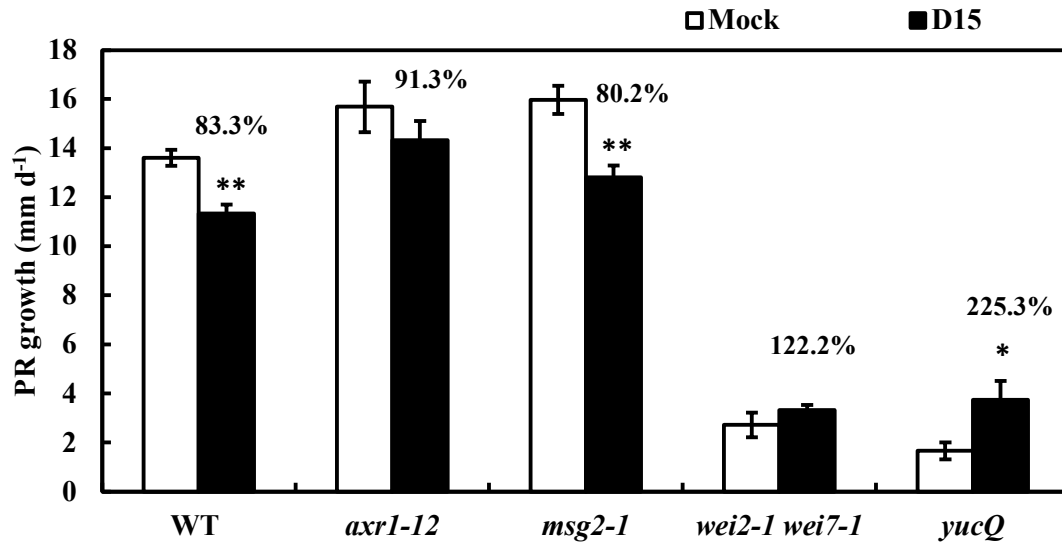


Figure 1.18 The resistance to carotenoid catabolism inhibitor D15 in auxin-related mutants.

Five-day-old plants of WT and auxin-related mutants were transferred to medium with or without 50 μ M D15. The PR growth rate was analyzed from 2 to 3 dat. The percentage means the relative PR growth rate in D15-treated plants compared to mock-treated plants. Data represent the means $\pm$ SE from 8 seedlings. *Significant differences compared to mock-treated plants (*: $P < 0.05$, **: $P < 0.01$; Student's t -test).

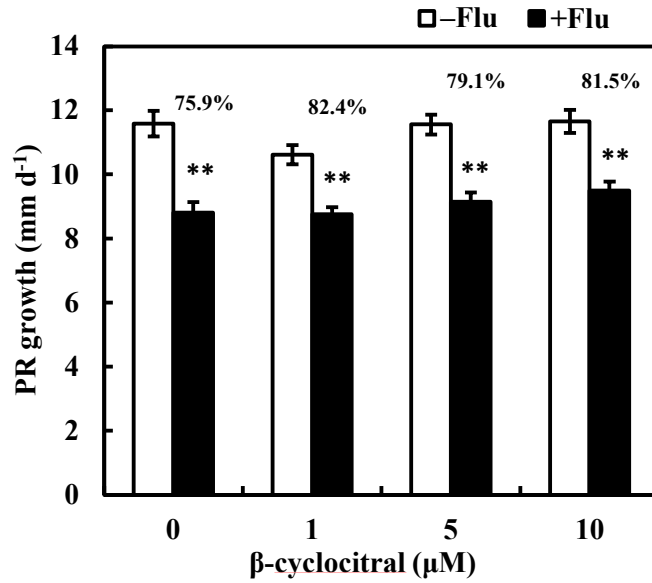


Figure 1.19 β -cyclocitral is not involved in carotenoid-mediated regulation of PR growth.

Five-day-old plants were transferred to medium with or without 800 nM fluridone and β -cyclocitral. The PR growth rate was analyzed from 1 to 2 dat. The percentage means the relative PR growth rate in fluridone-treated plants compared to non fluridone-treated plants. Data represent the means $\pm$ SE from 8 seedlings. *Significant differences compared to non fluridone-treated plants (**: $P < 0.01$; Student's t -test).

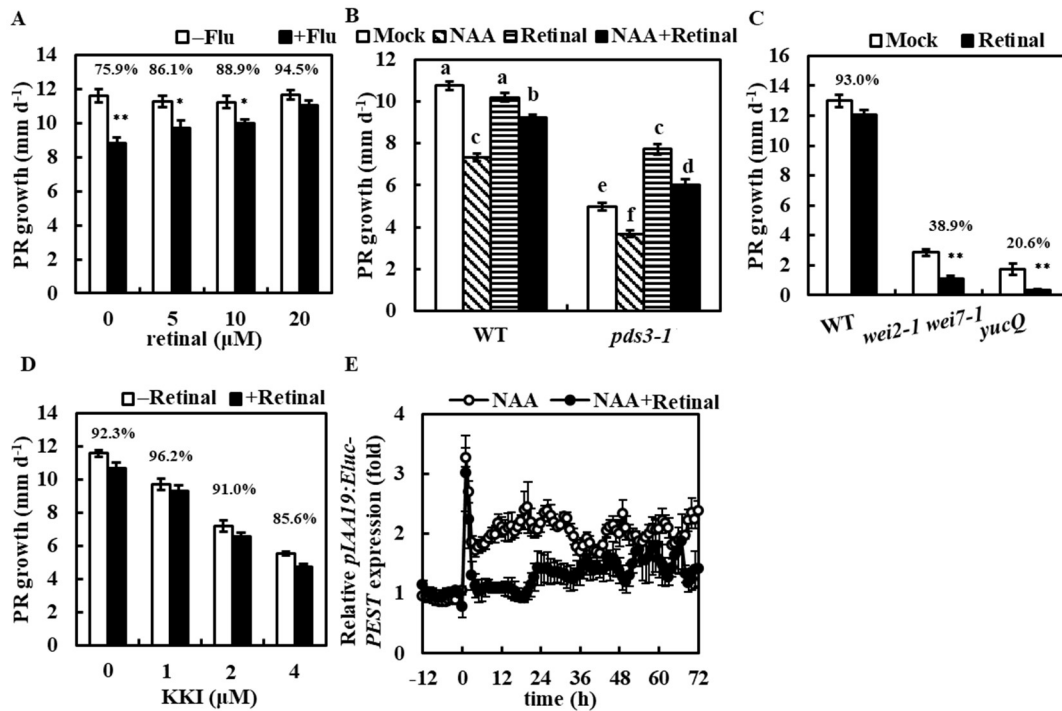


Figure 1.20 Retinal restores fluridone- and auxin-induced suppression of PR growth.

(A) Five-day-old plants were transferred to new medium with or without 800 nM fluridone and retinal. (B) Five-day-old plants of WT and *pds3-1* were transferred to new medium with or without 20 μM retinal and 20 nM NAA. (C) Five-day-old plants of WT and the auxin-deficient mutants were transferred to new medium with or without 20 μM retinal. (D) Five-day-old plants were transferred to new medium with or without 20 μM retinal and KKI. The PR growth rate was analyzed from 1 to 2 dat in (A) and 2 to 3 dat in (B–D). The bars in (B) indicate mock (white), 20 nM NAA (diagonal stripe black), 20 μM retinal (horizontal striped black) and NAA + retinal (black). The percentage means the relative PR growth rate in fluridone-treated plants compared to non fluridone-treated plants in (A) and the relative PR growth rate in retinal-treated plants compared to mock-treated plants in (C, D). (E) The *pLAA19::Eluc-PEST* expression. Four-day-old plants grown on 100 μM D-luciferin medium were transferred to new D-luciferin-containing medium for a 1 d preincubation. Then plants were transferred to new medium containing 100 μM D-luciferin, 10 nM NAA and with or without 20 μM retinal at time 0. Data represent the

2590 means $\pm$ SE from 8 to 10 seedlings in (A–D) and the means (relative to the means of
2591 time -1 h) $\pm$ SE from 6 seedlings in (E). Different lowercase letters above the bars in
2592 (A, C, D) indicate significant differences at $P < 0.05$ (one-way ANOVA following
2593 Tukey-Kramer test). *Significant differences compared to non fluridone-treated plants
2594 in (A) and to non retinal-treated plants in (C) (*: $P < 0.05$, **: $P < 0.01$; Student's t -
2595 test).

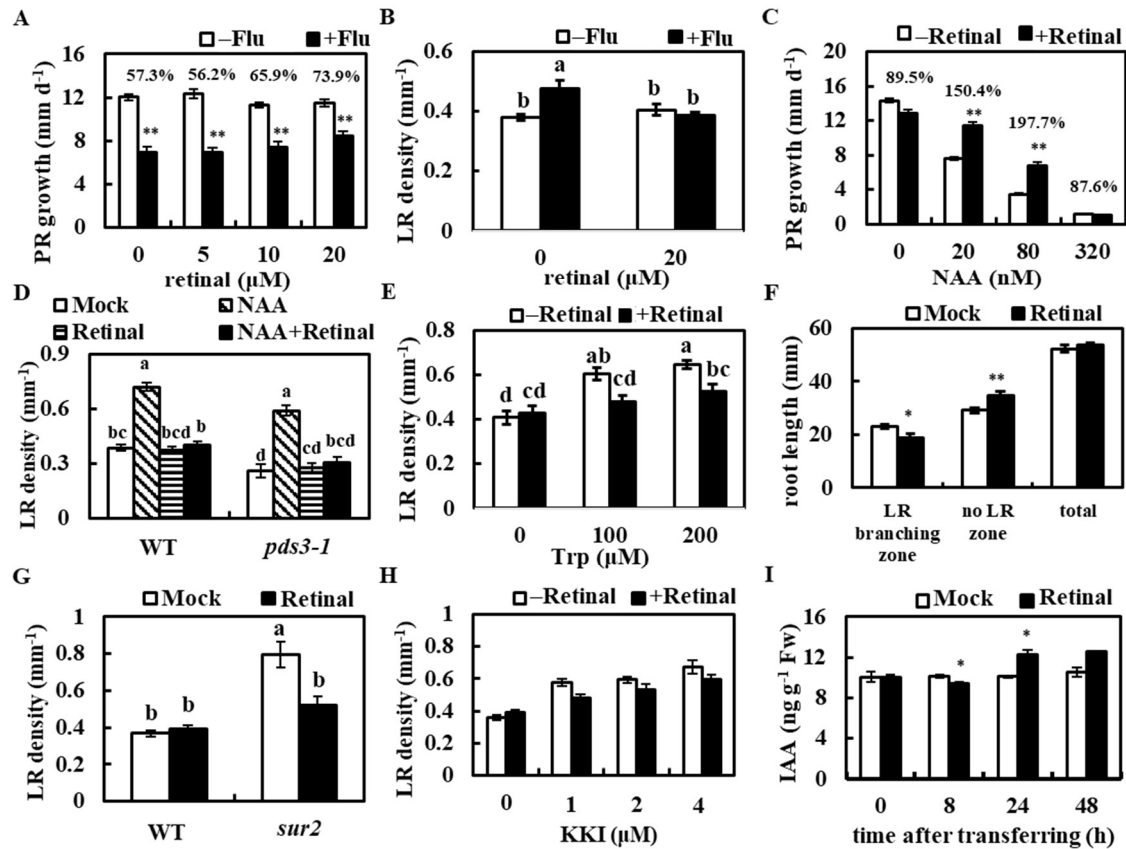
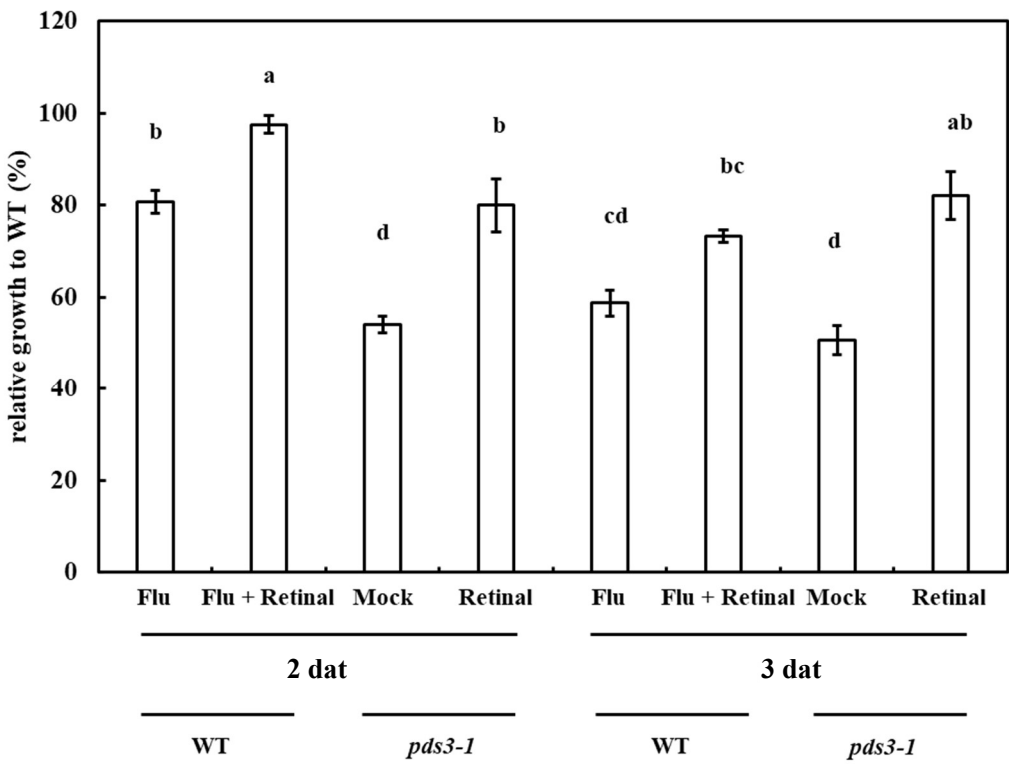


Figure 1.21 Retinal is responsible for the fluridone effect and regulates auxin levels and response.

(A, B) Five-day-old plants were transferred to medium with or without 800 nM fluridone and retinal. (C) Five-day-old plants were transferred to medium with or without 20 μM retinal and NAA. (D) Five-day-old plants of WT and *pds3-1* were transferred to medium with or without 20 μM retinal and 20 nM NAA. (E) Five-day-old plants were transferred to medium with or without 20 μM retinal and tryptophan. (F) The length of LR branching zones (from the root–shoot junctions to the youngest LRs toward the root tips), no LR zones (from the youngest LRs to the root tips) and total roots. Five-day-old plants were transferred to medium with or without 20 μM retinal. (G) Five-day-old plants of WT and *sur2* were transferred to medium with or without 20 μM retinal. (H) Five-day-old plants were transferred to medium with or without 20 μM retinal and KKI. The PR growth rate in (A, C) was measured from 2 to 3 dat. The root length in (F) was measured at 3 dat. The LR density in (B, D, E, G, H) was counted at 3 dat. The percentage means the relative PR growth rate in fluridone-

2612 treated plants compared to non fluridone-treated plants in (A) and the relative PR
2613 growth rate in retinal-treated plants compared to mock-treated plants in (C). The bars
2614 in (D) indicate mock (white), 20 nM NAA (diagonal stripe black), 20 μ M retinal
2615 (horizontal striped black) and NAA + retinal (black). (I) IAA quantification by retinal
2616 treatment. Five-day-old plants were transferred to medium with or without 20 μ M
2617 retinal at time 0. The IAA contents in the roots were measured at the indicated time
2618 points. Data represent the means $\pm$ SE from 8 to 10 seedlings in (A–H) and from 3
2619 biological replicates in (I). Different lowercase letters above the bars (A, B, D, E, G)
2620 indicate significant differences at $P < 0.05$ (one-way ANOVA following Tukey-
2621 Kramer test). *Significant differences compared to non fluridone-treated plants in (A)
2622 and to non retinal-treated plants in (C, F, I) (*: $P < 0.05$, **: $P < 0.01$; Student's t -
2623 test).



2625

2626 Figure 1.22 The different effect of retinal to fluridone-treated WT plants and
2627 *pds3-1* plants in root growth recovery.

2628 Five-day-old plants of WT were transferred to medium in mock treatment, 800 nM

2629 fluridone treatment and co-treatment of 800 nM fluridone and 20 μ M retinal. Five-

2630 day-old plants of *pds3-1* were transferred to medium with or without 20 μ M retinal.

2631 The relative PR growth rate compared to the mock-treated WT plants was analyzed

2632 from 1 dat to 2 dat or from 2 dat to 3 dat. Data represents the means $\pm$ SE from three

2633 independent technical replicates. Different lowercase letters above the bars indicate

2634 significant differences at $P < 0.05$ (one-way ANOVA following Tukey-Kramer test).

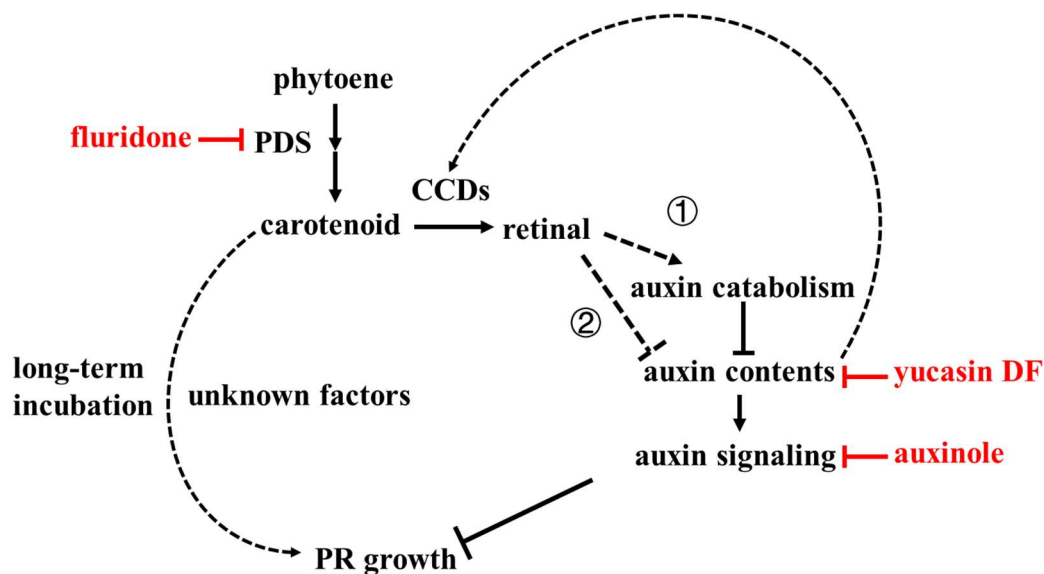


Figure 1.23 Putative model of carotenoid-mediated PR growth regulation.

Under normal conditions, PR growth is controlled by auxin homeostasis. The short-term fluridone treatment inhibits PDS activity and blocks consequent retinal biosynthesis to increase endogenous auxin levels. The increase of auxin levels may exceed its optimal levels for root growth. There are two possible pathways for retinal to regulate auxin contents and response. First is that retinal may accelerate the IAA catabolism pathway to reduce endogenous auxin levels. Second is that retinal may be involved in IPyA-independent auxin biosynthesis pathway (e.g., IG biosynthesis), so that decrease of retinal reduces precursors of auxin (e.g., tryptophan). Under long-term fluridone incubation, the carotenoid deficiency leads to an acceleration of root growth suppression via unknown factors. The activated auxin response feedback for the carotenoid catabolism gene CCD activity in the regulation of carotenoid content. Black dashed lines indicate unknown or predicted mechanisms.

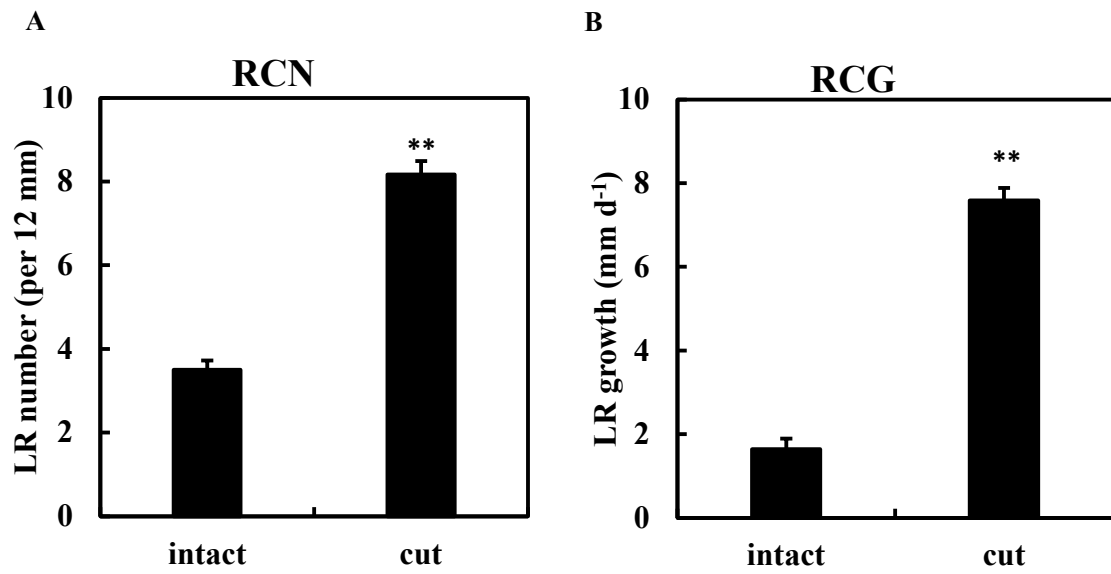


Figure 2.1 Root-cut induces LR number and growth.

Four-day-old plants were transferred to new medium and incubated for 1 d before root cutting. The LR number (A) was counted at 4 dac. The LR growth rate (B) was analyzed from 2 dac to 3 dac. Data represents the means $\pm$ SE from 12 seedlings. *Significant differences compared to intact plants (**: $P < 0.01$; Student's t -test).

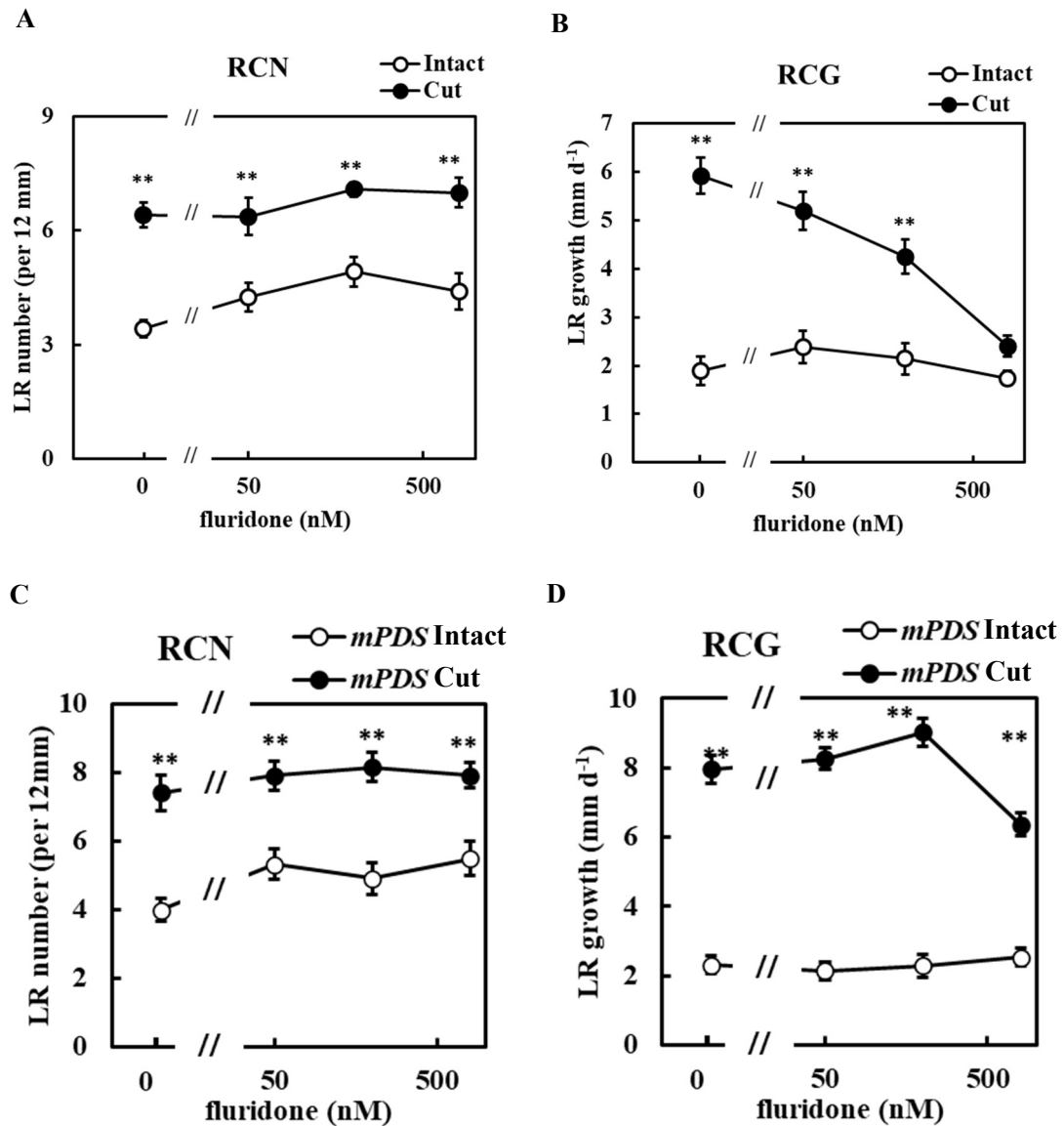
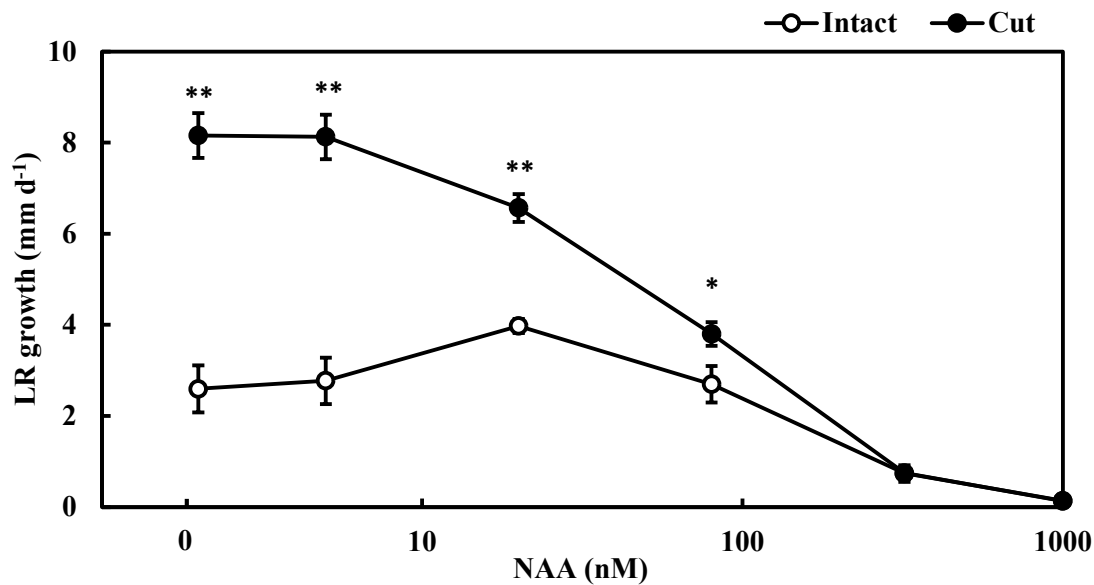


Figure 2.2 Fluridone specifically suppresses RCG via inhibiting PDS activity.

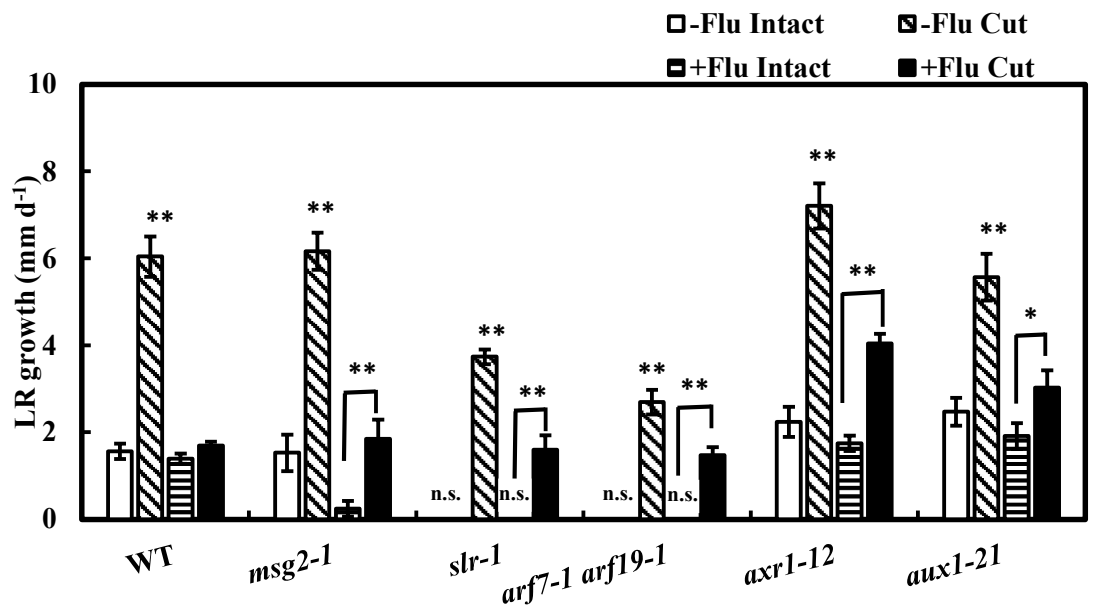
Four-day-old plants of WT and *mPDS* were transferred to new medium with or without fluridone and incubated for 1 d before root cutting. The LR number in WT (A) and *mPDS* (C) was counted at 4 dac. The LR growth rate in WT (B) and *mPDS* (D) was analyzed from 2 dac to 3 dac. Data represents the means $\pm$ SE from 12 seedlings.

*Significant differences compared to intact plants (**: $P < 0.01$; Student's *t*-test).

2693 A



2694 B



2695

2696 Figure 2.3 Fluridone suppresses RCG via the auxin pathway.

2697 (A) Four-day-old plants were transferred to new medium with or without NAA and
2698 incubated for 1 d before root cutting. (B) Four-day-old plants of WT and auxin signaling
2699 mutants were transferred to new medium with or without 800 nM fluridone and
2700 incubated for 1 d before root cutting. The LR growth rate was analyzed from 2 dac to 3
2701 dac. Data represents the means $\pm$ SE from 8 seedlings. *Significant differences
2702 compared to intact plants (*: $P < 0.05$, **: $P < 0.01$; Student's t -test). n.s. indicates no
2703 detectable data.

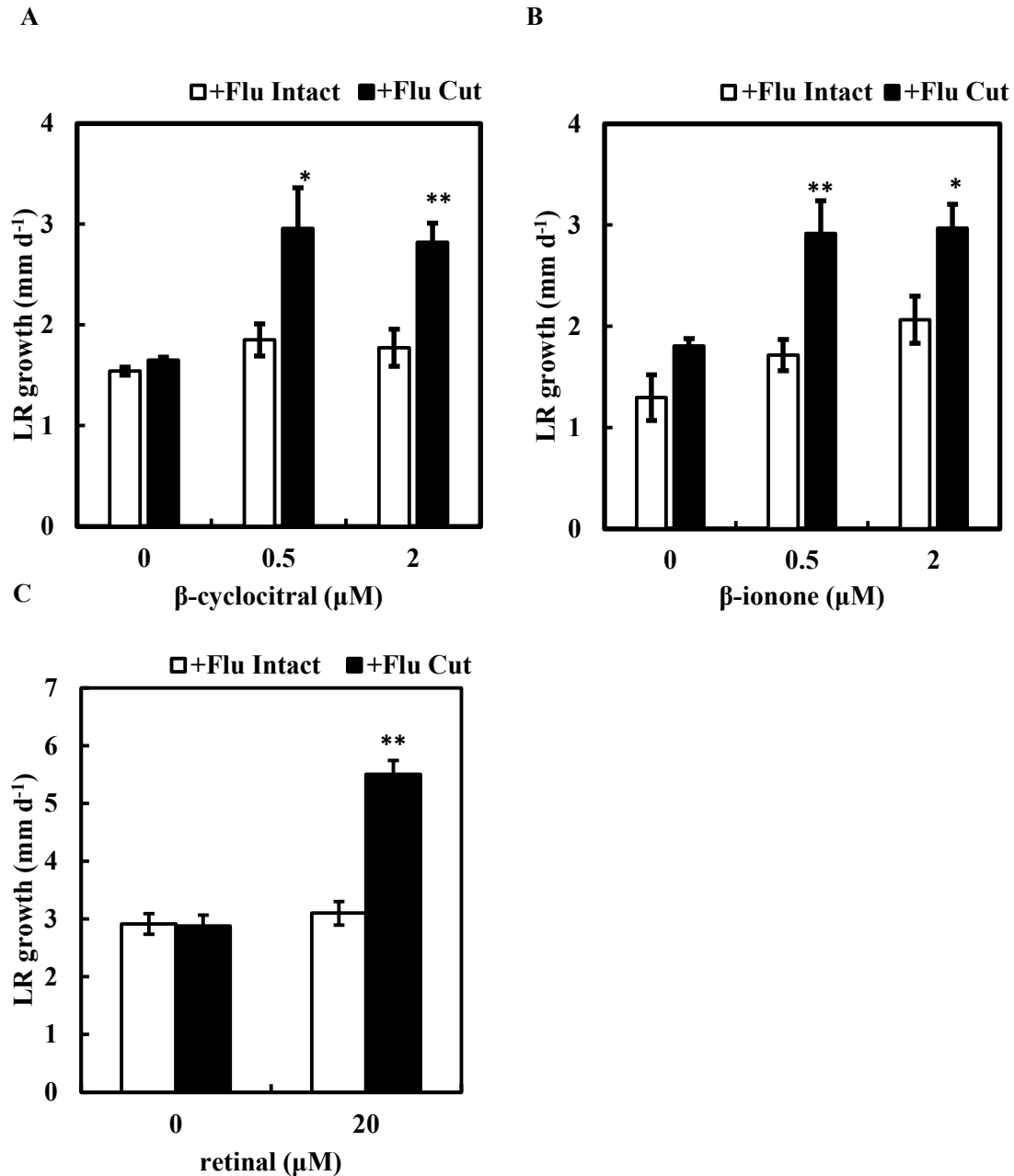


Figure 2.4 Apocarotenoids partially rescue fluridone-mediated RCG abolishment.

Four-day-old plants were transferred to fluridone-containing medium with or without apocarotenoids and incubated for 1 d before root cutting. The LR growth rate in β -cyclocitral treatment (A), β -ionone treatment (B) and retinal (C) was analyzed from 2 dac to 3 dac. Data represents the means $\pm$ SE from 8 seedlings. *Significant differences compared to intact plants (*: $P < 0.05$, **: $P < 0.01$; Student's t -test).

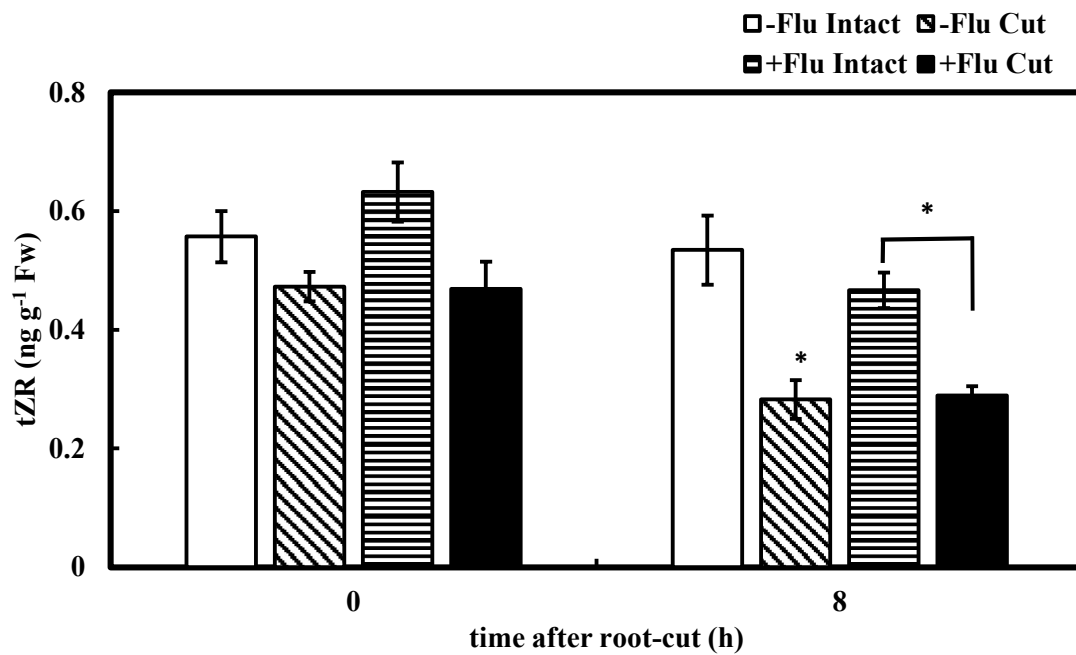


Figure 2.5 Root-cut reduces endogenous cytokinin levels.

Four-day-old plants were transferred to new medium with or without 800 nM fluridone and incubated for 1 d before root cutting. The tZR contents in roots were measured at the indicated time points. Data represents the means $\pm$ SE from 3 biological replicates. *Significant differences compared to mock-treated intact plants if not specifically indicated (*: $P < 0.05$; Student's *t*-test).

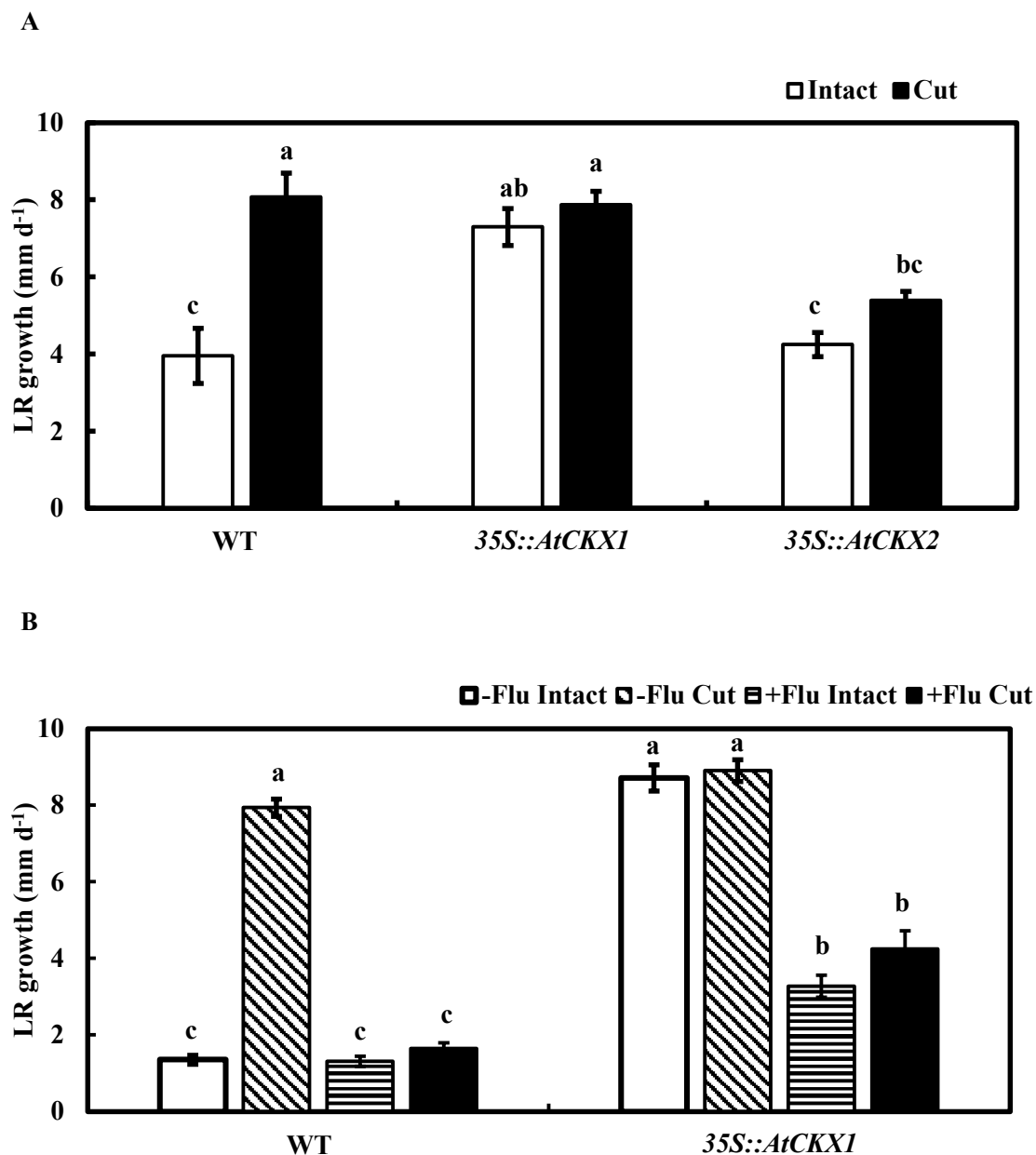


Figure 2.6 Cytokinins play a negative role in RCG regulation.

(A) Four-day-old plants of WT and *35S::AtCKXs* were transferred to new medium and incubated for 1 d before root cutting. (B) Four-day-old plants of WT and *35S::AtCKX1* were transferred to new medium with or without 800 nM fluridone and incubated for 1 d before root cutting. The LR growth rate was analyzed from 2 dac to 3 dac. Data represents the means $\pm$ SE from 8 seedlings. Different lowercase letters above the bars indicate significant differences at $P < 0.05$ (one-way ANOVA following Tukey-Kramer test).

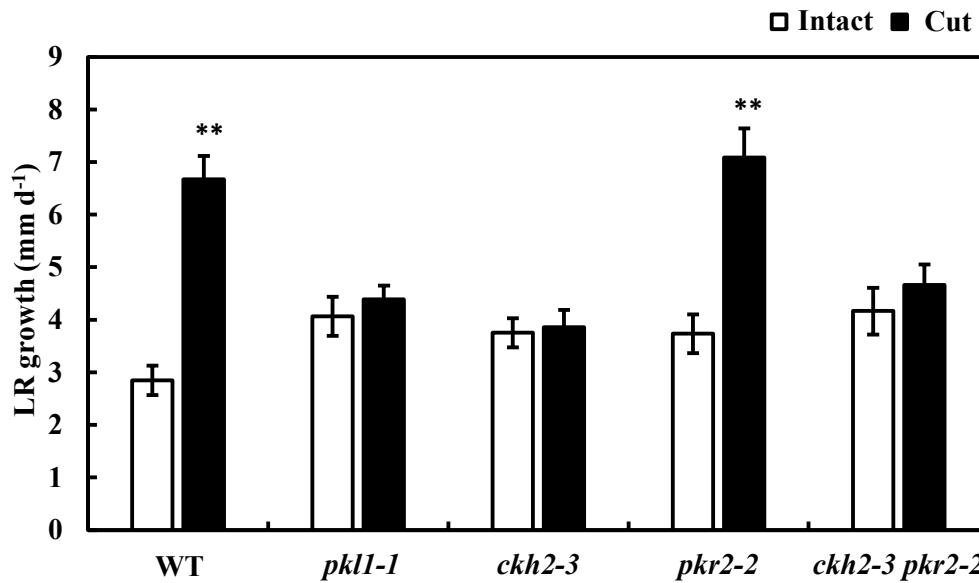


Figure 2.7 PICKLE is necessary for RCG.

Four-day-old plants of WT and *pk1* mutants were transferred to new medium and incubated for 1 d before root-cutting. The LR growth rate was analyzed from 2 dac to 3 dac. Data represents the means $\pm$ SE from 10 seedlings. *Significant differences compared to intact plants (**: $P < 0.01$; Student's *t*-test).

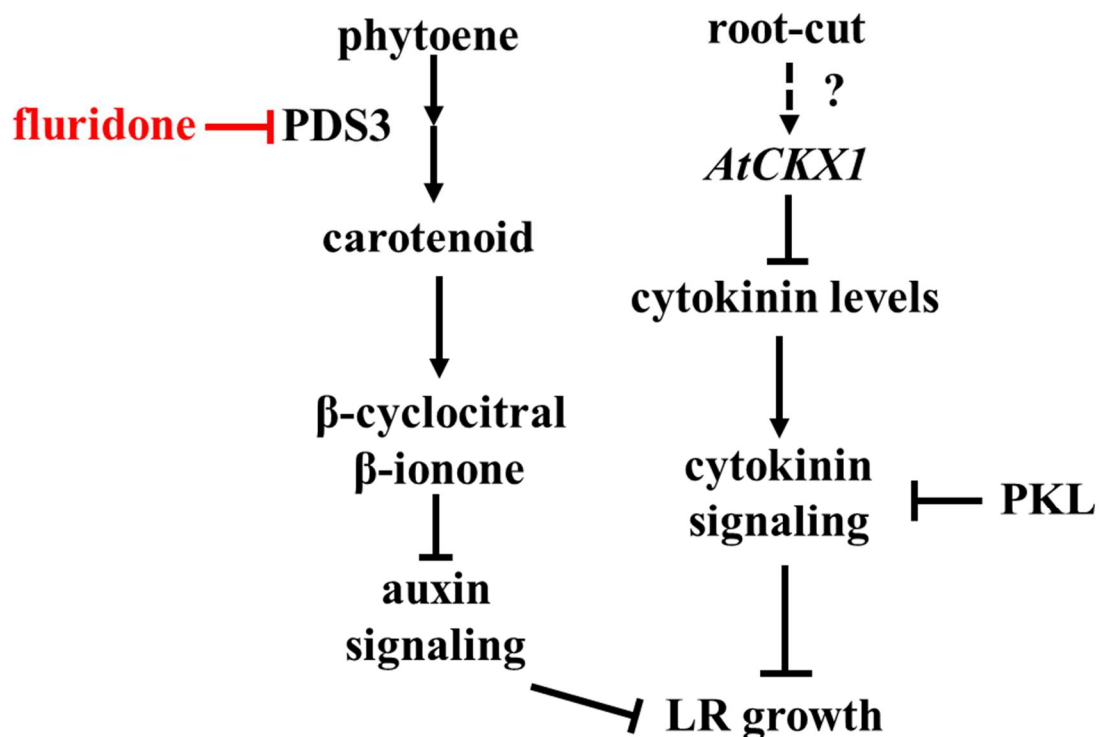


Figure 2.8 Putative model for fluridone- and cytokinin-mediated RCG regulation. Root-cut enhances LR growth by reducing endogenous cytokinin levels. Root-cut may reduce endogenous cytokinin levels via up-regulating *AtCKXs* or via unknown pathway. PKL may negatively regulate cytokinin response to regulate RCG. On the other hand, fluridone specifically suppresses root-cut-induced LR growth via the auxin pathway. The apocarotenoids play an intermediate role in this regulation. The interaction between the carotenoid and cytokinin pathway in RCG regulation needs to be clarified in the future.