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Molecular genetic study of lateral root regeneration on environmental stresses 「環境ストレスによる側根再生の分子遺伝学的研究」

A DISSERTATION

submitted to the Graduate School of Life Science, Hokkaido University in partial fulfillment of the requirements for the degree DOCTOR OF LIFE SCIENCE

by

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- (2) <u>Xu Dongyang</u>, Miao Jiahang, Yumoto Emi, Yokota Takao, Asahina Masashi, Watahiki Masaaki: 「*YUCCA*-mediated auxin biosynthesis and auxin transport are required for cut-induced lateral root formation in Arabidopsis」日本植物生理学会,第58回日本植物生理学会年会(平成29年3月,鹿児島)
- (3) <u>Xu Dongyang</u>, Watahiki Masaaki: 「YUCCA-mediated local auxin biosynthesis and auxin transport are required for cut-induced lateral root formation in Arabidopsis」 International Graduate Program, Training Program for Global Leaders in Life Science (IGP-GLSS), The 4th International Life-Science Symposium (平成 28 年 11 月, 札幌)
- (4) <u>Xu Dongyang</u>, Watahiki Masaaki: 「Root cutting induces lateral root formation through auxin signaling pathway」日本植物生理学会,第57回日本植物生理学会年会(平成28年3月, 岩手)
- (5) <u>Xu Dongyang</u>, Watahiki Masaaki: 「Lateral root formation of cut root which bypasses normal pathway of lateral root formation」International Graduate Program, Training Program for Global Leaders in Life Science (IGP-GLSS), The 3th International Life-Science Symposium (平成 27 年 11 月, 札幌)
- (6) <u>Xu Dongyang</u>, Watahiki Masaaki: 「Lateral root formation of cut root which bypasses normal pathway of lateral root formation」Molecular Plant, The 1st Molecular Plant International Symposium: From Model Species to Crops (平成 27 年 7 月, 上海, 中国)
- 4. 講 演 (その他)
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以 上

Abstract

Plants have a powerful ability of organ regeneration in response to adverse environmental conditions such as physical damage or exposure to toxic chemicals. A root system with the primary root (PR) injured is able to undergo regeneration by increases in lateral root (LR) number and acceleration of LR growth. This regeneration ability has been extensively used in agricultural or horticultural techniques such as "root pruning", where part of the root system was removed and a new root system with more branches and smaller size built, to control plant growth and fruit quality. However, how the damage of PR leads to the regeneration of LRs remains elusive. In this study, I try to answer this question by revealing the underlying mechanisms of LR regeneration in response to environmental stresses, particularly mechanical wounding such as root pruning or wound related peptide hormones, like *At*Peps.

In the first chapter of this thesis, genetic and molecular mechanism of root regeneration following root pruning was investigated. I found 1) After removal of the PR tip by root pruning, wild-type plants treated with polar auxin transport (PAT) inhibitors or auxin-signaling mutants that are defective in LRs recovered LR formation. 2) Induction of *IAA19* following root pruning indicates an enhancement of auxin signaling by root pruning. 3) Endogenous levels of IAA increased after root pruning and *YUCCA9* was identified as the primary gene responsible. 4) PAT-related genes were induced after root pruning and the YUCCA inhibitor yucasin suppressed root regeneration in PAT-related mutants. These results indicate the crucial role of *YUCCA9*, along with other redundant *YUCCA* family genes, in the enhancement of auxin biosynthesis following root pruning. This further enhances LR number.

In the second chapter of this thesis, the plant elicitor peptides, AtPeps, which has been extensively studied for their function on the innate immune response, however less documented in their role on plant growth and development, were investigated for their effect on root formation. In this study, I found 1) AtPep1 and AtPep2 inhibited PR growth, meanwhile increased LR density. 2) The PEPR2 receptor was responsible for the *At*Peps-induced root system morphology changes. 3) AtPep1 and AtPep2 inhibit PR growth through the disturbance of cell cycle in root tip, thus promote LR formation, which is dependent on the auxin signaling pathway. 4) This process is independent of AtPeps' role on pattern-triggered immunity, as flg22 and elf18 were unable to trigger the same response in the root. 5) AtPep1 treatment induced the ectopic expression of *PEPR1* and *PEPR2* in the root tip, which also occurred with mechanical damages on root tip, suggesting the potential role of AtPep1 in response to environmental stimuli, though the specific biological meaning is yet to be revealed. These results indicate that AtPep1 and AtPep2 modify the root system architecture through the inhibition of PR growth and promotion of LR formation, which is dependent on the auxin signaling pathway.

Root cutting removed the effect of PR tip on the remaining root tissue. Treatment of *At*Peps to the PR tip suppressed the PR growth. Both treatments represent a suppression of PR activity, which led to the increase of LR number and acceleration of LR growth. This reflects the plants' high plasticity to maintain a balanced growth of the root system architecture when part of the root is injured. These two ways of PR damage triggered the shared downstream pathways of auxin signaling and LR formation, however, the perception of the damage signals is differed. In this study, I showed how LR regeneration was induced by the restriction of PR growth, elucidated different mechanisms underlying this process with root pruning or root tip inhibition by *At*Peps, and discussed how different environmental stresses differentially induce LR regeneration through the common integrator of auxin.

Abbreviations

AFB, auxin-signaling F-box protein

ARF, auxin response factor

Aux/IAAs, auxin/indole-3-acetic acid

BUM, 2-[4-(diethylamino)-2-hydroxybenzoyl] benzoic acid

crane/iaa18, crane/indole-3-acetic acid18

GUS, β -glucuronidase

IPA, indole-3-pyruvate

JA, jasmonic acid

LAX, LIKE AUX1

LBD/ASL, lateral organ boundaries-domain/asymmetric leaves2-like

LR, lateral root

LRI, lateral root initiation

LRP, lateral root primordia

LRR, leucine-rich repeat

MAKR4, MEMBRANE-ASSOCIATED KINASE REGULATOR4

MAMPs, microbe-associated molecular patterns

MAPK, mitogen-activatedprotein kinase

MDR/PGP, multiple drug resistance/P-glycoprotein

MeJA, methyl jasmonate

MeSA, methyl salicylate

MS, Murashige and Skoog

msg2/iaa19, massugu2/indole-3-acetic acid19

NAA, 1-naphthaleneacetic acid

NPA, N-1-naph-thylphthalamic acid

PAMPs, pathogen-associated molecular patterns

PAT, polar auxin transport

PEPR1, PEP-Receptor1

PIN, PIN-FORMED

PR, primary root

PTI, pattern-triggered immunity

qRT–PCR, quantitative reverse transcription–PCR

RCG, root-cutting induced LR growth

RCN, root-cutting induced LR number

ROS, reactive oxygen species

RSA, root system architecture

RSJ, root-shoot junction

shy2/iaa3, suppressor of hy2 mutation2/indole-3-acetic acid3

slr/iaa14, *solitary root/indole-3-acetic acid14*

TAA1/SAV3, tryptophan aminotransferase of arabidopsis1/shade avoidance3

TAR, TAA related

TIBA, 2,3,5-triiodobenzoic acid

TIR1, transport inhibitor response1

WT, wild-type

YUC, YUCCA

General introduction

Organ regeneration is a distinctive feature of plants, which exists throughout their lifetime and contributes to their robustness in adverse conditions. *Arabidopsis thaliana* (Arabidopsis) as a eudicot has a taproot system, which is consist of an embryonic-developed (embryonic radicle derived) primary root (PR) and postembryonic-developed lateral roots (LRs). The root system architecture (RSA) is therefore both genetically determined and modified by different environmental conditions such as water availability, nutrient levels, physical obstacles, or damage (Al-Ghazi et al. 2003, Ditengou et al. 2008, Sena and Birnbaum 2010, Sugimoto et al. 2011, Van Norman et al. 2013). The high plasticity of RSA helps plants to adapt to an ever-changing environment.

The agricultural technique, root pruning is an application of RSA regeneration, where part of the root system is removed; this results in the production of better quality fruit (Schupp and Ferree 1987). It has also been used by horticulturists to control plant size or vigor, as occurs in the production of bonsai plants. The induction of LR formation by excision of the root tip or by root pruning has long been reported (Thimann 1936, Van Overbeek 1939, Torrey 1950, Wightman et al. 1980, Biddington and Dearman 1984). However the molecular mechanism remained elusive.

Root pruning on one hand causes mechanical damage to the PR, on the other hand restricts the further growth of the PR. In plants, when the shoot apex is removed, the growth of lateral buds is promoted. This well-known phenomenon is called apical dominance and is considered to be regulated by the plant hormone auxin: auxin is predominantly produced in the shoot apex and inhibits the growth of lateral buds through the auxin dependent production of strigolactones, once the apical shoot is removed, reduced auxin concentration lead to the growth of lateral buds (Booker et al. 2003, Brewer et al. 2009, Thimann and Skoog 1934). In RSA, induction of LRs by removal of the primer root may possess different mechanisms.

In addition to mechanical damages like root pruning, other factors also cause the restriction of PR growth. For example, in *Arabidopsis* seedlings being exposed to a NaCl gradient, primary growth was inhibited while LR emergence was promoted (Galvan-Ampudia and Testerink 2011). Among the factors restrict PR growth there is a peptide elicitor AtPep1, which is a peptide hormone related to the innate immune response of plants. Upon AtPep1 application, the PR growth was inhibited and the root biomass increased (Krol et al. 2010, Huffaker et al. 2006).

In this study, I examined the underlying mechanisms of LR regeneration in response to environmental stresses, particularly mechanical wounding like root pruning (root cutting) or chemicals like *At*Peps. I will characterize the shared or differed mechanisms of these different stimuli on modification of RSA. I also try to answer the yet unknown question how plants balance the growth of PR and LRs and how the inhibition of PR growth leads to the induction of LR formation.

Root system architecture

Conventional vascular plants develop roots, which possess vascular tissues like xylem and phloem. Roots can anchor the plant body to the soil, absorb water, minerals, and store photoassimilates, thus are important for plants' growth and development (Bellini et al. 2014). In different plant species, the root systems show variable morphologies which are called RSA. There are basically two types of RSA, the taproot system (or allorhizic system) in gymnosperms and dicotyledons like *Arabidopsis*, tomato (*Solanum lycopersicum* L.), carrot (*Daucus carota*), and poplar (*Populus spp.*), and the fibrous root system (or homorhizic system) in

monocotyledons such as maize (*Zea mays* L.), rice (*Oryza sativa* L.), onion (*Allium cepa*), garlic (*Allium sativum*), and tulip (*Tulipa spp.*) (Bellini et al. 2014). The taproot system is consist of a single thick central PR with thin or no LRs, the fibrous root system has a small and short-lived primary and adventitious roots derived from shoots, stems, or leaves (Bellini et al. 2014).

The morphology of RSA is largely dependent on the degree of branches, which further influences the efficiency of absorbing water and nutrients. The RSA is modified by environmental stimuli, thus represent the high plasticity and adaptation of plants in response to diverse growth conditions (Van Norman et al. 2013).

Auxin is a key regulator of LR formation

LRs are initiated from pairs of pericycle cells that possess developmental potential as plant stem cells. These pericycle cells are selected and directed to become LR founder cells and form LRs by both intrinsic and environmental signals (De Smet et al. 2007, Dubrovsky et al. 2008, Richter et al. 2009, Sugimoto et al. 2010). The phytohormone auxin plays fundamental roles in many aspects of plant growth and development and it is a key regulator of LR development (Fukaki et al. 2007, Lavenus et al. 2013). Auxin signaling is known to be essential for LR formation, it is an integrator of endogenous and exogenous signals for root branching (Casimiro et al. 2001, De Smet et al. 2006, Fukaki and Tasaka 2009, Lavenus et al. 2013), it begins with the degradation of a class of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) through TRANSPORT INHIBITOR RESPONSE1 (TIR1) auxin receptor (Dharmasiri et al. 2005, Kepinski and Leyser 2005), resulting in the activation of the AUXIN RESPONSE FACTOR (ARF) (Ulmasov et al. 1997, Nanao et al. 2014). ARF7 and ARF19 transcription factors that further induce the expression of downstream target genes like LATERAL ORGAN BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES2-LIKE (LBD/ASL) family genesLBD16/ASL18 and LBD29/ASL16 and induce LR initiation at the protoxylem-pole pericycle cells (Okushima et al. 2005, Okushima et al. 2007, Goh et al. 2012, Lee et al. 2009). AUX/IAA gain-of-function mutants such as massugu2/indole-3-acetic acid19 (msg2/iaa19) (Tatematsu et al. 2004), crane/indole-3-acetic acid18 (crane/iaa18) (Uehara et al. 2008), suppressor of hy2 mutation 2/indole-3-acetic acid3 (shy2/iaa3)(Tian and Reed 1999), and solitary root/indole-3-acetic acid14 (slr/iaa14) (Fukaki et al. 2002)are auxin insensitive and are defective in LR formation.

Polar auxin transport facilitates LR formation

Polar auxin transport (PAT), which mobilizes IAA from source to sink tissues, is facilitated by auxin influx carriers known as AUX1 and LIKE AUX1s (LAXs) and by auxin efflux carriers known as PIN-FORMEDs (PINs) and MULTIPUL DRUG RESISTANCE/P-GLYCOPROTEINS (MDR/PGPs) (Swarup and Péret 2012, Paponov et al. 2005, Blakeslee et al. 2005, Kramer and Bennett 2006). PAT, through these auxin transporters, collectively generates auxin gradients and maintains an auxin maximum, both of which are essential in LR formation and positioning (Casimiro et al. 2001, Marchant et al. 2002, Benková et al. 2003, De Smet 2012, Lavenus et al. 2013). *AUX1* promotes LR formation and *LAX3* promotes LR emergence (Marchant et al. 2002, Swarup et al. 2008). PIN-dependent local auxin gradients are considered to be an essential element for organ formation, and the dynamic rearrangement of PIN1 is correlated with the establishment of auxin gradients and lateral root primordia (LRP) development (Benková et al. 2003). MDR/PGPs and PINs define two distinct auxin efflux systems, but can interact physically and functionally to modulate auxin efflux, create auxin gradients, and regulate LR formation (Geisler et al. 2005, Lin and Wang 2005, Wu et al. 2007, Petrášek et al. 2006, Mravec et al. 2008). Consequently, the inhibition of PAT activity only by N-1-naph-thylphthalamic acid (NPA) is sufficient to block LR initiation (Casimiro et al. 2001).

Auxin biosynthesis pathway

Natural auxin, IAA, is mainly synthesized in a two-step pathway from tryptophan. First, tryptophan is converted to indole-3-pyruvate (IPA) by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/SHADE AVOIDANCE3 (TAA1/SAV3) family of aminotransferases, IPA is then converted to IAA by the *YUCCA* (*YUC*) family of flavin monooxygenases (Tao et al. 2008, Stepanova et al. 2008, Yamada et al. 2009, Won et al. 2011, Mashiguchi et al. 2011). Several lines of evidence have indicated that the IPA pathway is essential for auxin biosynthesis in *Arabidopsis*, and that the *YUC* family is a rate-limiting step in this pathway (Cheng et al. 2007, Stepanova et al. 2008, Zhao et al. 2001, Zhao 2012).

LR pre-patterning controlled by intrinsic developmental signals

The primary LR is initiated from the basal meristem of the PR, where root cap-derived auxin influences the amplitude of oscillatory gene expression in the basal meristem and the elongation zone of the root, which leads to the pre-patterning of LR initiation sites (Moreno-Risueno et al. 2010, Xuan et al. 2015). The pre-patterning process is marked by the expression of a series of genes, like *GATA23*, *MEMBRANE-ASSOCIATED KINASE REGULATOR4* (*MAKR4*), and *IAA19* (Xuan et al. 2015). In the basal meristem and elongation zone, *DR5::Luciferase* expression was observed to rhythmically pulse with a period of

 \sim 6 h, which matched with the period of LR pre-branch site production (Moreno-Risueno et al. 2010).

It is recently reported that the source of auxin is provided by the cyclic programmed cell death of root cap cells (Xuan et al. 2016, Möller et al. 2017). It is noteworthy that not all of the pre-branch sites emerge to become LRs (Moreno-Risueno et al. 2010). These dormant pre-branch sites may present a selective mechanism for LR formation under certain growth conditions.

LR formation induced by mechanical stresses

Mechanical forces are important regulators for plant morphogenesis. LRs always emerge from the convex side of PR binding, resulting in a left-right alternation of LRs. It has been shown that transient physical bending of 20 s in the PR was sufficient to elicit LR formation to the convex side of the curve, which is through triggering a Ca²⁺ transient within the pericycle (Richter et al. 2009). The bending-induced LR initiation was independent of an auxin supply from the shoot and was not disrupted in auxin transport or signaling mutants (Richter et al. 2009). Bending caused by gravitropic curvature also led to the initiation of LRs, where a subcellular relocalization of PIN1 was observed (Ditengou et al. 2008).

Release the pericycle cells from the restraints of adjacent endodermis by targeted single cell ablation of endodermal cells triggered the pericycle to re-enter the cell cycle and induced auxin-dependent LR initiation (Marhavý et al. 2016).

AtPeps as endogenous peptide defense signal in Arabidopsis

In animals, the pathogen-associated molecules that initiate the innate immunity are called pathogen-associated molecular patterns (PAMPs). In plants, these molecules are called elicitors and are released upon injury or infection (Huffaker et al. 2006). Some of the elicitors are peptides, including fungal elicitors Pep13, AVR9 (van den Ackerveken et al. 1993, Hahlbrock et al. 1995, Kamoun 2001), and elicitins, as well as bacterial elicitors hrpZ, NPP1, flg22, elf13, and elf18 (He et al. 1993, Fellbrich et al. 2002, Kunze et al. 2004, Navarro et al. 2004), all of which are derived from pathogens.

The peptide elicitor derived from the plant itself is called plant elicitor peptide (Pep). AtPep1, the first discovered Pep, was isolated from Arabidopsis leaves (Huffaker et al. 2006). It belongs to a family of eight members, among which AtPep1 and AtPep5 have been biochemically isolated from Arabidopsis, while other members were synthesized based on their homology to AtPep1 (Huffaker et al. 2006, Huffaker and Ryan 2007, Bartels et al. 2013, Yamaguchi and Huffaker 2011).

AtPep1 is a 23-aa peptide derived from the C terminus of a 92-ss precursor protein PROPEP1 without posttranslational modification (Huffaker et al. 2006). Its homologs AtPep2-8 are derived from the C terminus of their precursor proteins PROPEP2-8 respectively (Huffaker et al. 2006, Huffaker and Ryan 2007, Bartels et al. 2013). AtPep1 has the characteristics of an endogenous elicitor of the innate immune response, like alkalinizing the medium of suspension cultured cells, activating the transcription of the defense gene PDF1.2 (defensin), and inducing the production of H_2O_2 (Huffaker et al. 2006). It also strongly induces the expression of the precursor gene PROPEP1 at nanomolar concentration, the constitutive overexpression of which induces defense genes and confers resistance to a root pathogen *Pythium irregulare* (Huffaker et al. 2006).

The Pep family genes are-likely to be conserved in higher plants in both dicots and monocots, the maize homolog *Zm*Pep1 and the rice homolog *Os*Pep1 have been reported (Huffaker et al. 2011, Liu 2013).

Receptors of AtPeps

AtPeps have been reported to be perceived by two receptors, PEP-Receptor1 (PEPR1) and PEPR2, which are plasma membrane leucine-rich repeat (LRR) receptor kinases (Yamaguchi et al. 2006, Krol et al. 2010, Yamaguchi et al. 2010). Through photoaffinity labeling and binding assays in transgenic tobacco (*Nicotiana tabacum*) cells expressing *PEPR1* and *PEPR2* it was demonstrated that PEPR1 is a receptor for AtPep1-6 and PEPR2 is a receptor for AtPep1 and AtPep2 (Yamaguchi et al. 2010).

AtPep signaling in response to environmental stimuli

It has been reported that the expression *PROPEP1* was induced by environmental conditions or chemicals like wounding, ethephon, methyl jasmonate (MeJA), and methyl salicylate (MeSA) (Huffaker et al. 2006, Yamaguchi et al. 2010). The receptors PEPR1 and PEPR2 were also transcriptionally induced by wounding and MeJA (Yamaguchi et al. 2010).

Chapter 1

YUC mediated local auxin biosynthesis acts synergistically with auxin transport to regulate cut-induced lateral root regeneration in *Arabidopsis*

1.1 Introduction

LR formation induced by mechanical root damage has been documented in a wide range of plant species. To the best of our knowledge, the first report of root regeneration by root cutting documented aerial roots of tropical grape (*Vitis sp.*) (Zimmerman and Hitchcock 1935). Root pruning of wheat has also been reported to increase LR number and auxin content (Vysotskaya et al. 2001). Joshi et al. (2016) reported that root cutting or heat ablation of adventitious root cap enhanced *CYCLIN B1* expression in potato LRP and suggested that this occurred through the activation of auxin signaling. Although regeneration of the root system following injury is critical for the survival and fitness of sessile plants, the molecular mechanisms underlying this regeneration process are poorly understood. While root pruning is typically refer to an agricultural technique used on woody plants in the field, the cutting of roots under sterile conditions reflects the results of root pruning in the field. In this study, we report on the molecular mechanism of root regeneration following root cutting. We identified YUC9 as the primary gene responsible for elevation of IAA level by root cutting, and characterized the regulatory role of auxin biosynthesis and transport in this process.

1.2 Materials and methods

1.2.1 Plant materials and growth conditions

Arabidopsis thaliana mutants and WT plants used in this study were in the Columbia background except for *yuc5* which is in the Landsberg *erecta* background. Joanne Chory's laboratory provided *sav3-2* (Tao et al. 2008). *yuc1, yuc2, yuc3, yuc4, yuc5, yuc6, yuc7, yuc8, yuc9, yuc10* and *yuc11* mutants were obtained from the laboratory of Yunde Zhao (Zhao 2008, Cheng et al. 2007, Cheng

et al. 2006). Seeds of *shy2-101*, *slr*;and *crane* came from the laboratory of HidehiroFukaki (Goh et al. 2012, Fukaki et al. 2002, Uehara et al. 2008); *arf7 arf19* from Yoko Okushima (Okushima et al. 2005); *pin3-4* from Jiří Friml (Friml et al. 2003); *aux1-7* (Pickett et al. 1990) from the Arabidopsis Biological Resource Center; *pgp1-101* and *pgp1-101 pgp19-101* from Tatsuya Sakai (Nagashima et al. 2008); and *tir1-1 afb2-3* from Mark Estelle (Parry et al. 2009). Seeds were surface sterilized with chlorine gas at least for 30 min. Seeds were suspended in 0.3% agarose and sown on half strength of 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 2 d in the dark. Plants were grown on vertically oriented plates at 23°C under constant light conditions. Stock solutions of phytohormones and inhibitors were prepared in dimethyl sulfoxide and filtered through a 0.45-µm disc filter.

1.2.2 Root-cutting and quantification of LRP and LR number

Plants were grown on vertical plates for 4 d then transferred to new half-strength MS medium with or without inhibitors or auxin. After 1 d of pre-incubation, the root was cut 12 mm from the RSJ. Plant images were acquired with a flatbed scanner (GT-X980, EPSON) 4 d after root cutting. The number of emerged LRs within 12 mm from the RSJ was counted with ImageJ software (version 1.48, Rasband 1997-2016). To count the number ofLRP and LRs in Fig. 1.1E, Fig. 1.10F, and Fig. 1.3, roots were observed with a microscope (Nikon Eclipse, X20 and X40 PlanApo, Nikon instruments). LRP were counted between stage II and stage VII, defined according to Malamy and Benfey (1997). Stage I LRP were not counted for this study since the optical assessment of LRP could interfere with its

differentiation. The growth rate of the first LR was measured with time-lapse imaging using a digital camera (Lumix G4, Panasonic) with time-lapse instruments; acquired images were analyzed using ImageJ. pIAA19.::GUS plants (Kami et al. 2014) were subjected to the root cutting procedure described above and GUS histochemical analysis was conducted as described previously (Saito et al. 2007) with the exception of fixation and incubation times (0.5h).

1.2.3 RNA isolation and quantitative reverse transcription–PCR (qRT–PCR) analysis

Root samples were harvested at 0–11 mm or 0–2.5 mm (Fig. 1.5G) from the cut end of roots and frozen in liquid nitrogen. Total RNA was extracted and purified using a FavorPrep Plant Total RNA Mini Kit (Favorgen Biotech Corp.). cDNA was synthesized from total RNA according to the manufacturer's instruction (ReverTra Ace qPCR RT Master Mix with gDNA Remover, Toyobo). qRT–PCR was performed in optical 96-well plates with a LightCycler 480 II system (Roche Life Science), using KOD SYBR qPCR Mix (Toyobo). Primer pairs spanning the exon-exon junction were designed using the QuantPrime program (Arvidsson et al. 2008) to avoid genomic DNA amplification, as listed in Table 1.2 (Blacha 2009, Muto et al. 2007). The specificity of reactions was verified by melting 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 (Muto et al. 2007). The qRT–PCR program wasone cycleof 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 (Ct) value was determined by using the second derivative maximum method (Tichopad et al. 2003) in the LightCycler software (version 1.5, Roche Life Science). The data were analyzed using the comparative threshold

cycle (Ct) method $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008).

1.2.4 Quantification of IAA and JA

Root samples were harvested 1–12 mm from the RJS and frozen in liquid nitrogen. The hormone analysis was carried out as described previously (Enomoto et al. 2017, Miyamoto et al. 2016). Briefly, samplesof approximately 100 mg fresh weight were suspended in 80% (v/v) aqueous methanol with [13C6]-IAA, [2H2]-JA, and [13C6]-JA-Ile as internal standards. 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 (LC-ESI-MS/MS) composed of 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 was extracted from the MassHunter software (Agilent Technologies) and examined in Excel (Microsoft).

1.3 Results

1.3.1 Root cutting induces LR formation

To investigate root regeneration in *Arabidopsis*, the PR of 5-d-old seedlings was cut at 12 mm from the root-shoot junction (RSJ). In wild-type (WT) plants, the number of LRs on the remaining 12-mm-long root portion in root-cut plant increased subtly but significantly after 4 d in comparison with the 12-mm-long corresponding area in intact plant (Fig. 1.1A, B). This increase in LR number in root-cut versus intact plants was more evident in the auxin-signaling mutant *msg2-1* (Fig. 1.1A, B), which is a dominant mutant of *AUX/IAA19*, where the

number of LRs in the intact plants was lower than in intact WT plants. In addition to the increase in LR number, the cutplants of both WT and msg2-1had longer LRs than the intact control (Fig. 1.1A). We measured the growth rate of the first LR proximal to the RSJ in cut plants and demonstrated that it grew more than two times faster than the intact control in both WT and *msg2-1*(Fig. 1.1C). Additionally, after 4 d recovery, the total length of the root system in root-cut WT and *msg2-1* plants was the same as in the corresponding intact controls (Fig. 1.1D). We named the root-cutting induced increase in LR number RCN (<u>root-cutting</u> induced increase in LR <u>n</u>umber) and the acceleration of LR growth RCG (<u>root-cutting</u> induced increase in LR growth). In this study, we focus on RCN. Other dominant AUX/IAA mutants and loss-of-function ARF mutants were also examined (Table 1.1). These mutants all showed RCN with different degree, with the exception of *slr* where the RCN did not occur within 4 d of root cutting. We did, however, find that a longer subsequent incubation of 16 d or exposure to high temperature (28 °C) induced RCN in *slr* (Fig. 1.2E–H), confirming a previous notion LR was occasionally induced in *slr* when the PR were cut off(Fukaki et al. 2002). It is interesting that *arf7 arf19* formed several LRs at the cut end, and *shy2* formed short LRs throughout the PR following root cutting (Fig. 1.2B–D).To investigate LR initiation and LRP development during the RCN in more detail, the number of LRP and LRs within 12mm from the RSJ was determined. In intact WT plants, 5-d-old seedlings (0 h in Fig. 1.1E) have approximately one LR and 4 LRP per seedling. With incubation, the LRP number decreased and new LRs emerged (Fig. 1.1E). The total number of LRP and LRs in the 12-mm area was constant in 0 h to 4 d in intact plant, indicating that few new LRP formed after the 5-d-old seedling stage in this area (Fig. 1.1E). Compared with intact plants, the total number of LRP and LRs in root-cut plants was higher than in intact plants

24 h after treatment, indicating that new LRPs emerged 8–24 h after root cutting (Fig. 1.1E). At 4 d after incubation, almost all of the LRP developed to LRs and few LRP remained in the 12-mm area of interest in both intact and root-cut plants (Fig. 1.1E). To investigate the spatial pattern of LRs and the PR, LR number was counted in the region proximal to the cut end (0–6 mm from the cut end) and the region distal to the cut end (6–12 mm from the cut end). LR induction was evident in the 0–6 mm area (Fig. 1.1F) in plants with root cutting. Furthermore, increases in the total number of LRP and LRs were observed in the 0–1.24 mm area from the cut end (Fig. 1.3).These results indicate that RCN occurs proximal to the cut end of the root.

1.3.2 Root cutting activates auxin signaling

As auxin signaling triggers LR initiation and development (De Smet et al. 2006, Fukaki and Tasaka 2009); the involvement of auxin signaling in RCN was examined. The expression of an early auxin-inducible gene Aux/IAA19(Tatematsu et al. 2004), auxin-inducible transcription factor ARF19 (Okushima et al. 2005, Okushima et al. 2007), and its downstream gene LBD29 (Lee et al. 2009) increased after root cutting (Fig. 1.4A–C). Expression of Aux/IAA19 reached a peak 4 h after root cutting (Fig. 1.4A). Notably, activation of IAA19 expression was evident in cut end, as shown by β -glucuronidase (GUS) staining (Fig. 1.4D). LR formation related genes LBD16 and LBD18 (Fig. 1.5A, B), and auxin efflux career genes PIN1, PIN3, and PIN7 (Fig. 1.5C–E), which are known to respond to auxin (Vieten et al. 2005), were also induced by root cutting. These results suggest that root cutting induces LR formation through activating auxin signaling pathway.

1.3.3 The RCN requires auxin biosynthesis and PAT activity

As auxin transport is important for the accumulation of auxin that promotes LR formation (Lavenus et al. 2013), we employed a PAT inhibitor, NPA (Fujita and Syono 1996, Casimiro et al. 2001), to study the role of auxin transport in RCN. NPA suppressed LR formation seriously in intact plants, however, surprisingly, root cutting recovered LR formation even in plants treated with high concentration (10 μ M) of NPA that completely abolished LR growth in intact plants (Fig. 1.6A). NPA also abolished LR formation in *msg2-1* intact plants, however root cutting still induced LR formation (Fig. 1.6A). We further examined different MDR/PGP-specific PAT inhibitors; inhibitor 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid (BUM) (Kim et al. 2010), and 2,3,5-triiodobenzoic acid (TIBA) (Geldner et al. 2001). Like NPA, root cutting restored LR formation under the effect of either BUM or TIBA (Fig. 1.6B, C). These results indicate that RCN is resistant to PAT inhibition to a certain extent. The robust nature of the RCN led us to hypothesize that auxin biosynthesis is involved in RCN. Besides auxin transport, *de novo* auxin biosynthesis is another means by which auxin accumulates (Lavenus et al. 2013). L-kynurenine was identified as an auxin biosynthesis inhibitor that targets TAA1 and its related enzymes TAA RELATEDs (TARs) (He et al. 2011). Moderate (10 µM) to high concentrations (50 μ M) of L-kynurenine abolished RCN, resulting in root-cut plants with a similar number of LRs as the intact control (Fig. 1.6D). Yucasin is an auxin biosynthesis inhibitor that targets YUCCA flavin-containing monooxygenase, which is downstream of TAA1/TARs (Nishimura et al. 2014). Nishimura et al. (2014) reported that the inhibitory effect of yucasin on auxin biosynthesis is restricted in WT plants but more obvious in *sav3-2* mutant. In WT plants, although yucasin decreased LR number in both intact and root-cut plants

in a concentration-dependent manner, the number of LRs was higher in root-cut plants, indicating the occurrence of RCN (Fig. 1.6E). However, the LR number of root-cut*sav3-2* was comparable with intact *sav3-2* plants with high concentrations of yucasin (50 μ M and 100 μ M), indicating a defect in the RCN mechanism (Fig. 1.6E). These results suggest that auxin biosynthesis plays an essential role in RCN. We also attempted to block both PAT and auxin biosynthesis at the same time to determine the robustness of RCN. The combination of yucasin and NPA completely blocked LR formation in both intact and root-cut WT plants (Fig. 1.6F). Taken together, these results suggest that auxin biosynthesis is the primary factor for RCN, while both auxin biosynthesis and PAT activities together are necessary for the maximum RCN.

1.3.4 Root cutting elevates the endogenous IAA level

The requirement of auxin biosynthesis for RCN led us to measure the endogenous IAA level in roots. IAA increased 2 h after root cutting in both WT and msg2-1 (Fig. 1.7), with a higher IAA level found in both intact and root-cut msg2-1 plants than in WT plants. Then we hypothesize that RCN is activated through the elevation of endogenous IAA level following root cutting. To confirm this hypothesis, we applied the exogenous auxin, 1-naphthaleneacetic acid (NAA) and found that LRs were actually induced with a dose-dependent manner in both WT and msg2-1 (Fig. 1.8). Induction of LRs in msg2-1 suggests that elevation of auxin level can overcome the auxin insensitivity and LR deficiency in msg2-1.

1.3.5 YUC9 is responsible for induction of auxin biosynthesis in RCN

Yucasin treatment in *sav3-2/taa1*, which reduces the substrate for YUCCAs, reduced the RCN (Fig. 1.6E), suggesting a major role of *YUC* genes in RCN. All

available *yuc* mutants were examined and *yuc9* was found to have reduced RCN (Fig. 1.9). Although LR number in the intact *yuc9* was similar to intact WT, RCN of root-cut *yuc9* plants was reduced significantly compared with root-cut WT plants in control medium; this reduction was even more pronounced in NPA medium (Fig. 1.10A–C). This is consistent with previous results showing that RCN was completely abolished by a combination of NPA and yucasin (Fig. 1.6F). These results, in combination with previous observations that YUC9 is expressed in root tissue (Hentrich et al. 2013, Chen et al. 2014), led us to characterize YUC9 further. We then studied the gene expression of YUC9 and found that YUC9 expression was transiently affected by root cutting, increasing 2 h after root cutting before decreasing to the basal level after 12 h (Fig. 1.10D). Notably, the activation of YUC9 preceded the induction of AUX/IAA19 by 2 h (Fig. 1.4A). We then examined the endogenous IAA level. Although *yuc9* had a higher level of IAA than WT in intact plants, the level of IAA was not increased following root cutting in yuc9 as it did in the WT (Fig. 1.10E). Finally, LR initiation and LRP development were examined in *vuc9*, this confirms that RCN was lower 4 d after root cutting in *yuc9* than in WT (Fig. 1.10F). The expression level of TAA1/SAV3 was also examined. Although the increase of TAA1/SAV3 expression was not evident in whole roots (Fig. 1.5F), it became more obvious near the cut end (2.5 mm within cut end) (Fig. 1.5G); however this induction was later than the expression peak of IAA19, ARF19, LBD29, PIN1, and PIN7 (Fig. 1.4A–C; Fig. 1.5C, E), suggesting that TAA1/SAV3 plays a complementary role in RCN. Taken together, these results indicate that YUC9 is a key gene for RCN. Since the phytohormone jasmonic acid (JA) has been reported to be implicated in YUC9 mediated auxin biosynthesis in wounded leaves in Arabidopsis (Hentrich et al. 2013), we examined the role of JA in RCN. The methyl jasmonate (MeJA)

increased LR number in both intact and cut plant in WT, but not in *yuc9* (Fig. 1.11A). However, induction of JA or JA-Ile level was not found following root cutting (Fig. 1.11B, C).

1.3.6 Redundancy of YUC family genes involved in RCN

While there was a significant reduction in RCN in *yuc9* compared with WT, some RCN was still observed in this mutant, suggesting that there may be functional redundancy in the *YUC* gene family. To examine this hypothesis, different concentrations of yucasin were applied in conjunction with NPA, and further reduction of RCN in *yuc9* was observed (Fig. 1.12A). The sensitivity of *yuc9* to yucasin suggests that other yucasin-sensitive enzymes are involved in RCN in the *yuc9* mutant; these enzymes are likely to be members of the YUC family. Subtle reduction of RCN on *yuc6* was noticed but did not show significant difference to WT (Fig. 1.12C, D). The *yuc6* was introgressed into *yuc9*, and we observed further reduction of RCN in *yuc6 yuc9* especially in the presence of NPA (Fig. 1.12C, D). These results confirmed the gene redundancy among *YUC* gene family on RCN.

1.3.7 Synergistic effect of auxin biosynthesis and PAT activity on RCN

Pharmaceutical inhibition of both PAT and auxin biosynthesis influences RCN (Fig. 1.6F; Fig. 1.12A) and *yuc9* or *yuc9 yuc6* show high sensitivity to NPA (Fig. 1.10C; Fig. 1.12A, B, D). These results suggest a synergistic effect of PAT and auxin biosynthesis on RCN. We, therefore, examined RCN in auxin transport mutants under yucasin treatment. As in WT plants, LR number increased in PAT-related mutants *aux1*, *pin3-4*, *pgp1*, and *pgp1/pgp19* in control medium following root cutting. The number of LRs in these plants was, however, reduced in the presence of yucasin in both intact and root-cut plants. Conversely, yucasin

treatment did not affect LR number in either root-cut or intact WT plants (Fig. 1.13A). This indicates that these mutants were more sensitive to yucasin than WT plants, and suggests that RCN requires PAT activity, especially with the restriction of auxin biosynthesis. Next, the degree of RCN in WT and *yuc9* under NPA and TIBA treatment was further measured; here, both NPA and TIBA suppressed RCN more strongly in *yuc9* than in WT (Fig. 1.13B, C), indicating *yuc9* is more sensitive to PAT inhibitors than WT plants. These results indicate that PAT and auxin biosynthesis activity work synergistically, and the deficiency of PAT activity or auxin biosynthesis activity can be compensated by each other in RCN.

1.4 Discussion

Root pruning is a horticultural technique that is widely used in both the agricultural industry and by hobby gardeners. In this study, we investigated root cutting as a model system for root pruning. Root cutting induced two visible physiological responses, an increase in the LR number (RCN) and acceleration of LR growth (RCG). There are three potential explanations for RCN; first, root-cutting-induced signal directly activates the auxin signaling pathway; second, that auxin transport is enhanced by root cutting; and third, that auxin biosynthesis is enhanced by root cutting, contrary to the first hypothesis (Table 1.1). While PAT inhibitors inhibited LR formation in intact plants, root cutting was able to compensate for the reduction in PAT activity by inhibitors. Abolishment of RCN by auxin biosynthesis inhibitors suggests that auxin biosynthesis is the primary factor regulating RCN (Fig. 1.6D, E). Furthermore, we

showed that *YUC*-mediated auxin biosynthesis is responsible for RCN, and it cooperates with PAT to facilitate the maximum RCN.

1.4.1 YUC9 and YUC gene family members redundantly mediate cut-induced auxin biosynthesis

YUC family of flavin monooxygenases are the enzymes for the final step of IPA auxin biosynthesis pathway in Arabidopsis (Won et al. 2011, Mashiguchi et al. 2011, Zhao 2012). YUC1, YUC2, YUC4, and YUC6 were suggested to be mainly responsible for auxin biosynthesis in shoots, while YUC3, YUC5, YUC7, and YUC8 are responsible for this process in roots (Won et al. 2011). Auxin synthesized in roots but not that transported from shoots is necessary for normal root development (Chen et al. 2014). Increase of YUC9 expression in roots suggests that auxin biosynthesis following root cutting occurs in the root (Fig. 1.10D). Together with mutant analysis (Fig. 1.10B, C, F) and endogenous IAA quantification (Fig. 1.10E), YUC9 is suggested to be a key gene in RCN. In Nicotiana attenuata leaves, the induction of YUC-like genes by herbivore attack or wounding has previously been reported (Machado et al. 2016). In Arabidopsis leaves, expression of YUC9 has been shown to increase following wounding (Hentrich et al. 2013). In Arabidopsis leaf explants, involvement of YUC genes in adventitious root formation were reported (Chen et al.2016). In this study, functional redundancy of other YUC family members was investigated. Although only yuc9 showed significant reduction of RCN among yuc mutants in control medium, yuc6 and yuc7 also showed significant reduction of RCN in presence of NPA (Fig. 1.9A, B), suggesting other members of the *YUC* family are involved in RCN. Further reduction of RCN in *yuc9* by application of yucasin(Fig. 1.12A) and lower RCN in the *yuc6 yuc9* double mutant than in the *yuc9* single mutant also support this hypothesis (Fig. 1.12B–D). These results together with previous studies reveal the differential roles of *YUC* family genes in different biological contexts, indicating the tissue specificity and functionally redundancy of these genes.

1.4.2 Root cutting modifies the developmentally controlled LR patterning

Developmentally controlled LR initiation in intact plants was suggested to start from specific pericycle cells that gain the competency to become founder cells soon after they leave the basal meristem (Dubrovsky et al. 2000, Moreno-Risueno et al. 2010). After removal of the root tip, however, we found new initiation of LRs from mature regions of the PR (Fig. 1.1A, B, E) especially near the cut end (Fig. 1.1F; Supplementary Fig. 1). This new initiation represents reprogramming of pericycle cells to be LR founder cells, which involves dedifferentiation of pericycle cells and activation of cell cycles in pericycle cells (Dubrovsky et al. 2000, Ferreira et al. 1994, Malamy and Benfey 1997, Himanen et al. 2002, Beeckman et al. 2001). Auxin has been suggested to serve as a local morphogenetic trigger to specify LR founder cells (Dubrovsky et al. 2008). Exogenous auxin can reprogram pericycle cells to become LR founder cells in mature regions of the root (Blakely et al. 1988, Laskowski et al. 1995) (Fig. 1.8). In this study, root cutting triggered new initiation of LRs in the WT plants (Fig. 1.1E). Except for *slr*, the auxin signaling mutants used in this study all showed RCN under standard root cutting conditions. Even in slr, RCN did occur with a longer post-cutting incubation period or with exposure to high temperatures (Fig. 1.2E–H), indicating the robustness of the RCN response that change the programmed patterning of LRs. It is noteworthy that after root cutting, arf7 arf19, which has severely impaired LR formation (Okushima et al. 2005), formed LRs proximal to the cut end,

suggesting new initiation event occurred in the cut end (Fig. 1.2C, D). shy2, which increases the number of LR initiation sites but LRP remains in dormant (Goh et al. 2012), formed high density but relatively short LRs throughout the PR after root cutting (Fig. 1.2B), suggesting root cutting promoted the emergence of the dormant LRP in *shy2*. In the *AUX/IAA19* dominant mutant *msg2-1*, fewer LRs were present on intact roots in comparison with WT (Fig. 1.1A, B) (Tatematsu et al. 2004); root cutting, however, resulted in a similar number of LRs in WT and msg2-1 (Fig. 1.1A, B). Exogenous auxin also induced LR emergence in msg2-1 plants (Fig. 1.8). These results suggest that msg2-1 possesses auxin sensitivity but that sensitivity threshold has been heightened by the dominant AUX/IAA19/MSG2 mutation such that the endogenous IAA level is not strong enough to promote LR formation. Root cutting in *msg2-1*, however, promoted IAA biosynthesis sufficiently to overcome the heightened sensitivity threshold to auxin in msg2-1, to promote LR emergence (Fig. 1.7; Fig. 1.8). Taken together, root cutting modified the LR patterning through inducing LR initiation and promoting LR development in not only the WT plants but also the auxin signaling mutants.

1.4.3 Involvement of root tip in RCN

Ditengou et al. (2008) reported that gravity-induced bending of the *arf7 arf19* root relocated PIN1 protein in protoxylem cells at the first stage of lateral root initiation (LRI). As LRs did not subsequently emerge from the *arf7 arf19* root it has been suggested that *ARF7* and *ARF19* were not required for the first stage of LRI but were necessary for the later stages of LR development. Manual removal of the root tip after root bending however resulted in the emergence of LRs from the bending site of the *arf7 arf19* root, suggesting that there is an unknown mobile signal from root tip which suppress LR emergence (Ditengou et al. 2008). In the

present study, root cutting alone was able to induce LR formation in *arf7 arf19* (Table 1.1, Fig. 1.2C), suggesting that removal of root tip suppression signal promotes both the initiation and emergence of LR. In previous studies, the PR tip has also been proposed to have an inhibitory effect on LR formation (Zimmerman and Hitchcock 1935, Ditengou et al. 2008).Genetic ablation of the root cap cell reduced PR growth and increased the total number of LRs (Tsugeki and Fedoroff 1999), further suggesting the presence of a mobile signal from the root tip that suppresses LR formation. Conversely, auxin in the outer root cap cells was considered to be required for LR formation in intact plants (Xuan et al. 2015, Van Norman 2015). These contrasting observations suggest that root tip has both a promotional and an inhibitory effect on LR formation under different contexts; the mechanism regulating this process requires further investigation.

1.4.4 Auxin biosynthesis and auxin transport cooperatively regulate RCN

Auxin biosynthesis plays a critical role in RCN. *YUC9* as a primary gene responsible for RCN was induced 2 h after root cutting (Fig. 1.10D), preceding the induction of auxin signaling gene *IAA19* (Fig. 1.4A). The inhibition effect of PAT inhibitors was compensated by root cutting (Fig. 1.6A–C). This compensation is likely to have been mediated by activation of auxin biosynthesis as well as the increase in the level of PAT-related gene expression (*PIN1*, *PIN3*, and *PIN7*) following root cutting (Fig. 1.5C–E). Since *PIN1*, *PIN3*, and *PIN7* gene expression was able to be upregulated by auxin treatment in root (Vieten et al. 2005), we suggest that the observed increases in the expression level of these PAT-related genes resulted from the elevation of IAA level after root cutting (Fig. 1.5C–E). Induction of *TAA1/SAV3* plays a complementary role in cut-induced auxin
biosynthesis by providing the substrate of *YUC9*, IPA (Fig. 1.4A, C; Fig. 1.5C, E–G). Inhibition of both PAT activity and auxin biosynthesis totally abolished the RCN (Fig. 1.6F), indicating that they act synergistically. PAT-related mutants, pin3-4, aux1, pgp1, and pgp1/pgp19 showed higher sensitivity to yucasin than WT, with LR number decreased in both intact and cut plant following yucasin treatment (Fig. 1.13A). Auxin biosynthesis mutant yuc9 was more sensitive to auxin transport inhibitors than WT (Fig. 1.13B, C). These results indicate that auxin transport and auxin biosynthesis work together and may compensate for each other in RCN, however, when one of them is defective, the other will become more essential and sensitive to affect RCR.

1.4.5 Model for cut-induced LR formation

To date, how mechanical damages like root cutting regulate the regeneration of root systems and modify the number and placement of LRs to form a new RSA are largely unknown. In this study, we provide a model for root-cutting-induced auxin biosynthesis through primarily the activation of YUC9 and other YUC family genes. This results in the elevation of endogenous auxin level that induces PAT-related gene expression and enhances PAT activity before activating downstream auxin signaling genes and inducing RCN (Fig. 1.14). This model clarifies a previously unknown link between root cutting and YUC9 induction. While the signal activating YUC9 and regulating the response to root cutting is yet to be characterized, it is plausible that this signal is strongest at the cut end and spreads upward to the whole root. Further investigations are necessary to reveal the missing link between cut-induced signal and the activation of YUC-mediated auxin biosynthesis.

Chapter 2

AtPep1 and AtPep2 regulate lateral root formation through inhibition of root apical meristem

2.1 Introduction

The role of AtPeps on the innate immune response has been extensively studied (Huffaker et al. 2006, Huffaker and Ryan 2007), however their role on plant growth and development are less documented. In this study, I focus on the effect of AtPeps on the regulation of root growth. The results demonstrated that through the perception of PEPR2, AtPep1 and AtPep2 inhibit PR growth through the disturbance of cell cycle in root tip, thus promote LR formation, which is dependent on auxin signaling pathway. This process is independent of AtPeps' role on pattern-triggered immunity (PTI), which is also induced by flg22 and elf18.

2.2 Materials and methods

2.2.1 Plant materials and growth conditions

AtPeps were a kind gift from Dr. Yube Yamaguchi (Osaka Prefecture University). Flg22 and elf18 were a gift from Dr. Takeo Sato (Hokkaido University). Arabidopsis thaliana mutants and WT plants used in this study were in the Columbia background. Joanne Chory's laboratory provided sav3-2 (Tao et al. 2008). yuc1, yuc2, yuc3, yuc4, yuc6, yuc7, yuc8, yuc9, yuc10 and yuc11 mutants were obtained from the laboratory of Yunde Zhao (Zhao 2008, Cheng et al. 2007, Cheng et al. 2006). Seeds of shy2-101, and shcame from the laboratory of HidehiroFukaki (Goh et al. 2012, Fukaki et al. 2002, Uehara et al. 2008); arf7 arf19 from Yoko Okushima (Okushima et al. 2005); tir1-1 afb2-3 from Mark Estelle (Parry et al. 2009); AtproPep10x376, AtproPep10x3131, AtproPep10x4236, and AtproPep10x4244 from Dr. Yube Yamaguchi; pepr1-1, pepr2-1, and pepr1-1 pepr2-1 were from Dr. Yube Yamaguchi; and pPEPR1::GUSand pPEPR2::GUS were from Dr. Thomas Boller (University of Basel). Seeds were surface sterilized with chlorine gas at least for 30 min. Seeds were suspended in 0.3% agarose and sown on half strength of 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 2 d in the dark. Plants were grown on vertically oriented plates at 23°C under constant light conditions. Stock solutions of phytohormones and inhibitors were prepared in dimethyl sulfoxide and filtered through a 0.45-µm disc filter.

2.2.2 Chemical treatment and quantification of LR number

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 pre-incubation, Chemicals (AtPeps, flg22, elf18, or oryzarin) were applied to whole root (5µL), 0–12mm from RSJ (4µL), or root tip (1µL). For the application of chemicals to different regions of the root, the medium was cut to make a 1 mm gap between treated and untreated regions to avoid the diffuse of chemicals in the medium. Plant images were acquired with a flatbed scanner (GT-X980, EPSON) 4 d after root cutting. The number of emerged LRs was counted with ImageJ software (version 1.48, Rasband 1997-2016).

2.2.3 GUS staining

Plants of *pIAA19::GUS*, *pPEPR1::GUS* and *pPEPR2::GUS* were subjected to the *At*Pep1 treatment procedure described above and GUS histochemical analysis was conducted as described previously (Saito et al. 2007) with the exception of fixation and incubation times (30 min for *pIAA19::GUS* and *pPEPR2::GUS*, 20 min for *pPEPR1::GUS*). Roots were photographed using a microscope (Nikon Eclipse, X20 and X40 PlanApo, Nikon instruments) in combination with a camera (Panasonic DMC-G2).

2.3 Results and discussion

2.3.1 AtPep1 modifies the RSA through inhibition of PR and LR growth and increase of LR density

It was reported that the Arabidopsis plants constitutively overexpressing PROPEP1 and PROPEP2 increased root biomass in potting soil with or without inoculation with the pathogen P. Irregular (Huffaker et al. 2006), suggesting that AtPeps may have a role on plant growth and development besides the well-known role as an elicitor on PTI. In order to elucidate the role of AtPeps on root growth, I applied AtPep1 on the root of Arabidopsis, and found that LR density increased dose dependently (Fig. 2.1A, B), in contrast to the decrease of PR length (Fig. 2.1A, C). This change of root growth is also evident in the presence of NPA (Fig. 2.1D, E). The growth rate of PR decreased significantly after the application of AtPep1, suggesting AtPep1 has a negative effect on PR growth. I further examined seedlings overexpressing PROPEP1 and all of the four lines of plants showed an increase of LR density (Fig. 2.1G).

2.3.2 Effect of AtPep1 on root growth is mediated by PEPR2

AtPeps have been reported to be perceived by two plasma membrane receptors PEPR1 and PEPR2 (Krol et al. 2010, Yamaguchi et al. 2006, Yamaguchi et al. 2010). Both receptors trigger similar defense response associated with MAPK phosphorylation, ethylene production, and seedling growth inhibition (Bartels et al. 2013). The *pepr2-1* mutant was insensitive to AtPep1 treatment with regard to the changes of LR density and PR length, while *pepr1-1* mutant responded similar to WT, suggesting the increase of LR density and inhibition of PR growth is mediated by PEPR2 (Fig. 2.2A–D).

The inhibition of root length by AtPep1 in *pepr1* mutant while not in *pepr2* mutant was also observed in a previous report (Krol et al. 2010). PEPR2 was considered to play a major but not exclusive role in AtPep1-mediated inhibited of root elongation, which is fully dependent on extracellular Ca²⁺ [Ca²⁺]_{ext}, and is caused by the over-accumulation of amino acids in root cells by suppressing the expression of the AtGDU-mediated amino acid export pathway (Ma et al. 2014). PEPR1 and PEPR2 may control different downstream pathways of AtPep1-modulated genes in the root were fully dependent on *PEPR2* (Ma et al. 2014), suggesting the potential roles of *PEPR2* in regulating the root growth and development.

PEPR1 is the receptor for AtPep1-6 and PEPR2 is for AtPep1 and AtPep2 (Bartels et al. 2013, Yamaguchi et al. 2010). The PEPR1 and PEPR2 confer overlapping expression patterns, which are in the vascular tissue of roots and leaves while not in root tips (Bartels et al. 2013). Notably, the expression of *PEPR2* was more restricted to the central cylinder of the root, in contract to that of *PEPR1* in most root tissues (Bartels et al. 2013). The pericycle cell in the central cylinder is the site of LR initiation (Dubrovsky et al. 2008). The activity of *PEPR2* in central cylinder may reflect its association with LR formation.

2.3.3 AtPep2 has similar effect as AtPep1 on root growth

AtPep family consists of eight homologous peptides, which are derived from the C-terminal of the precursor proteins PROPEPs (Huffaker et al. 2006, Huffaker and Ryan 2007, Bartels et al. 2013). In this study, effect on root growth was examined with AtPep1, AtPep2, AtPep3, AtPep4, AtPep5, and AtPep6. Among the six members, AtPep2 increased LR density and inhibited PR length, which is

similar toAtPep1 (Fig. 2.3A, B). However other AtPeps showed no effect on root growth (Fig. 2.3A, B). The effect of AtPep2 is also perceived by PEPR2, since *pepr2-1* was insensitive to AtPep2 treatment while *pepr1-1* responded similar to WT (Fig. 2.3C, D).AtPep1-6 have been confirmed to be able to bind to PEPR1, activate alkalinization, and trigger mitogen-activatedprotein kinase (MAPK) phosphorylation (Yamaguchi et al. 2006, Bartels et al. 2013), however their effect on root growth are different, reflecting the potential differential roles of the AtPeps in regulating plant growth and development other than the defense response. Since AtPep3, AtPep4 and AtPep6 were synthesized, their failure to affect root growth may be also because the predicted peptides were not fully active.

2.3.4 AtPep1-induced LR formation is dependent on auxin signaling pathway

AtPep1 induced IAA19 expression proximal to the root tip (Fig. 2.4A), suggesting auxin signaling was enhanced by AtPep1. LR density of auxin related mutants in response to AtPep1 was examined. The dominant mutant of AUX/IAAs, msg2-1and shy2-3, increased LR density with AtPep1 treatment (Fig. 2.4B, C), consistent with our previous study that LR formation in msg2-1 and shy2-3 can be recovered by increase of auxin level (Fig. 1. 2A, B, D). The slr mutant, which is severely defective in LR formation (Fukaki et al. 2002), did not increase LR after AtPep1treatment (Fig. 2.4C). arf7 arf19 which is also able to produce LR with increase of auxin level (Fig. 1.2C, D), produced LR after AtPep1 treatment (Fig. 2.4B, C). The auxin perception mutant tir1 afb2-3 was less sensitive to AtPep1 (Fig. 2.4C), suggesting AtPep1-induced LR formation is dependent of auxin signaling pathway. Increment of LR density was less in auxin biosynthesis mutant yuc6, yuc9, and yucQ, wei2, wei7, and wei2 wei7 (Fig. 2.4D, E), suggesting AtPep1 increased LR density through activation of these auxin biosynthesis genes. Increment of LR density is completely abolished by the YUCCA inhibitor yucasin (Fig. 2.4F), confirming the role of auxin biosynthesis in AtPep1-induced LR formation. However, no significant elevation of auxin level in whole root was detected with AtPep1 treatment (Fig. 2.4G), suggesting local auxin biosynthesis while not the overall elevation of auxin level contributes to the increase of LR density.

2.3.5 JA signaling is involved in AtPep1-induced LR formation

The defence-related hormone JA was considered to be tightly connected to PEPR-mediated signaling (Yamaguchi and Huffaker 2011, Bartels and Boller 2015). JA related mutants were less sensitive of AtPep-triggered responses like reactive oxygen species (ROS) production, ethylene production, and seedling growth inhibition (Flury et al. 2013). AtPeps application elevated JA level slightly around 1 h after treatment (Flury et al. 2013), while JA synthesis induced by herbivore oral secretions was reduced in *pepr1 pepr2* mutant (Klauser et al. 2015). In this study, the JA biosynthesis mutant *jar1* and the perception mutant *jin1* and *jaw-1D* is less sensitive to AtPep1-induced LR formation (Fig. 2.5), suggesting JA signaling is involved in this process.

2.3.6 LR promotion is downstream of PR inhibition

AtPep1 application increased LR density and inhibited PR growth (Fig. 2.1A–F). In order to elucidate the correlation of these two effects, mutants with reduced LR density increment with AtPep1 treatment were measured for PR length. Among these mutants, the receptor mutant *pepr2-1* and *pepr1-1 pepr2-1* were insensitive to PR inhibition (Fig. 2.6). All of the auxin related mutants reduced PR length significantly with AtPep1 treatment (Fig. 2.6). Interestingly, JA related mutant *jar1* while not *jin1* and *jaw-1D* was insensitive to PR inhibition (Fig. 2.6). From these results, I suggest that *jar1* is upstream of PR inhibition, while *jin1, jaw-1D* and other auxin related mutants were downstream of PR inhibition but upstream of LR promotion. The special role of *jar1* among the JA related mutants in mediating AtPep1-triggered PR inhibition need to be further investigated. I further hypothesize that perception of AtPep1 through PEPR2 inhibited PR growth, which is mediated by *jar1*. Inhibition of PR growth triggered the activation of auxin biosynthesis through auxin biosynthesis genes like *YUC6*, *YUC9*, *WEI2*, and *WEI7*, then promoted the formation of LR through auxin signaling pathway, which is mediated by TIR1 and AFBs.

2.3.7 Root tip is the target tissue of AtPep1

Since AtPep1 inhibited PR and LR growth, we suggested the root tip is the response site to AtPep1. We also noticed that the induced LR formation in auxin mutants $msg2\cdot1$, shy2, and arf7 arf9 by AtPep1 was more pronounced near the root tip (Fig. 2.4B), suggesting root tip possesses higher sensitivity to AtPep1. AtPep1 was applied to different area of the root: whole root, 0–12mm from RSJ, or root tip. Interestingly, AtPep1 treatment on 0–12mm from RSJ did not change LR density, while treatment on root tip increased LR density similar to the whole root treatment (Fig. 2.7 A, B), suggesting the root tip is the primary responsive area for AtPep1. With regard to PR length, treatment on root tip inhibited PR growth similar to whole root treatment, while treatment on 0–12mm from RSJ suppressed PR length slightly (Fig. 2.7 A, C). When I observed the length of LR, surprisingly, although treatment on whole root and 0–12mm from RSJ suppressed 1st LR length, treatment on root tip on the contrary, increased 1st LR length (Fig. 2.7 A, D), suggesting that AtPep1's effect on PR tip not only promoted LR

initiation in upper part of the root, but also promoted the growth of LR. It is possible that AtPep1 entered the root through younger root tissues like PR tip or LR tip, where the Casparian strips that inhibited the entrance of substances into endodermal and vascular tissues are less developed (Alassimone et al. 2010, Naseer et al. 2012). With whole root treatment, newly emerged LR tips were exposed to AtPep1, so the LR length was suppressed, in contract to the root tip treatment where LR length was rather increased (Fig. 2.7D).

2.3.8 Ectopic expression of PEPR1 and PEPR2 in root tip was induced by AtPep1 The expression patterns of *PEPR1* and *PEPR2* promoter-GUS lines showed that these two genes expressed in the root excluding the root tips (Fig. 2.8A, B, Bartels et al. 2013). However, surprisingly, when AtPep1 was applied to the root tip, strong GUS staining was induced in the root tips of both *PEPR1* and *PEPR2* promoter-GUS lines ((Fig. 2.8A, B). The staining was more restricted to the central cylinder in *pPEPR2::GUS* lines. In *pPEPR1::GUS* line, where the expression was present in most root tissues in control condition (Bartels et al. 2013), interestingly, the expression was also more restricted to the central cylinder near the root tip. I suspect that the ectopic expression of *PEPR1* and PEPR2may present the potential of plants in response to particular growth conditions or environmental stimulus. The biological meaning of this response needs to be further investigated. When *At*Pep1 was applied to 0–12mm from RSJ, only weak induction of *PEPR1* and *PEPR2* expression was observed (Fig. 2.8C, D), suggesting the upper part tissues of the root is less sensitive to AtPep1 compared with the root tip. This supports our observation that application of AtPep1 on root tip increased LR density and suppressed PR length, while treatment on 0–12mm from RSJ less worked (Fig. 2.7 A–C).

2.3.9 Inhibition of PR growth through the disturbance of cell cycle in root tip by AtPep1 results in the increase of LR density

Since PR growth was inhibited by AtPep1, I suspect that root apical meristem activity may be suppressed. Seedlings expressing *pCYCLIN B*::*GUS*, the marker of cell cycle transition from G2 to M phase, were used. With AtPep1 application, a smaller meristem with strongly enhanced *pCYCLIN B*::*GUS* expression was observed (Fig. 2.9A), suggesting that cell cycle was disturbed with AtPep1 treatment. The mitosis inhibitor oryzalin also enhanced *pCYCLIN B*::*GUS* expression in the root apical meristem (Fig. 2.9B). Interestingly, when we observe the growth of seedlings in the presence of oryzalin, the RSA of the plants was similar to that of AtPep1 treatment: shorter PR and higher density and longer LR (Fig. 2.7A; Fig. 2.9C–E), suggesting that disturbance of cell cycle in root apical meristem suppressed PR elongation and increased LR density and growth.

2.3.10 Effect of AtPep1 on root growth is independent of the role of bacterial elicitor as flg22 and elf18

MAMPs like the bacterial flagellin (active epitope flg22) and elongation factor Tu (active EF-Tu epitopes elf13, elf18, and elf26) trigger the PTI response (Boller and Felix 2009, Bartels et al. 2013). AtPeps as the elicitors from plant, trigger PTI response similar to these bacterial elicitors (Bartels et al. 2013). I suspect whether AtPep1's effect on root growth regulation is also through the similar mechanism as PTI response. However, flg22 and elf18 showed no obvious effect on root growth as AtPep1 (Fig. 2.10A–C), suggesting the root growth regulation effect of AtPep1 is distinct from its role in PTI. In a previous report, AtPep1 was also shown to have a more pronounced inhibition effect on root growth compared with MAMPs (Krol et al. 2010).

2.3.11 PEPR1 and PEPR2 expression was induced by different abiotic stresses in root

Regarding that *At*Pep1 induced ectopic expression of *PEPR1* and *PEPR2* in root tip, I suspect there might be other environmental stimulus that affect the expression pattern of *PEPR1* and *PEPR2*. Abiotic stresses like salt, press, and cut were applied to the root tip of *Arabidopsis* seedlings.

Interestingly, salt treatment in root tip induced *PEPR1* and *PEPR2* expression in the mature zone rather than the root tip (Fig. 2.11A, B). *PEPR1*-s expression was in most root tissues while *PEPR2*s expression was more restricted to the central cylinder (Fig. 2.11A, B). *At*Pep1 is related to salt tolerance. The expression of *PROPEP1*, *PEPR1*, and *PEPR2* in the root was strongly induced by salt (Kilian et al. 2007). The *pepr2* mutant was reported to be salt tolerance (Colette et al. 2011). However, in this study *pepr1-2 pepr2-2* was more sensitive to salt (Fig. 2.12). The association of *At*Pep1 perception with salt tolerance needs further study.

Mechanical stresses like press, cut at meristem, or cut at elongation zone triggered ectopic expression of *PEPR1* and *PEPR2* in root tip (Fig. 2.11C–H). In meristem or elongation zone cut condition, *PEPR1*expression was also more restricted to the central cylinder (Fig. 2.11E, G), different from its expression under normal condition (Bartels et al. 2013). Wounding of leaves using forceps led to the induction *PROPEP1*, *PROPEP2*, *PROPEP3*, *PROPEP5*, and *PROPEP8* promoters in the vasculature (Bartels et al. 2013). In *Arabidopsis* leaf, mechanical wounding caused by cork borer did not induce the expression of *pPEPR1::GUS* or *pPEPR1::GUS*, however exposure to generalist or specialist feeding insects did trigger *pPEPR1::GUS* or *pPEPR1::GUS* expression (Klauser et al. 2015).

Either salt or mechanical damages represent adverse growth conditions for plants. Change of the expression patterns, especially the ectopic expression of *PEPR1* and *PEPR2* reflect a potential role of the receptors and AtPeps in response to these adverse conditions. Whether these environmental stimuli are associated with AtPep1-regulated root growth need to be further studied.

2.3.12 Model of AtPep1 and AtPep2-induced inhibition of PR elongation and promotion of LR formation

As shown in Fig. 2.13, in the root tip, exogenous applied AtPep1 and AtPep2 are perceived by PEPR2 and induce the ectopic expression of PEPR2 in root tip, which leads to the disturbance of the normal cell cycle marked by the induction of *CYCLIN B* expression. This results in the inhibition of PR elongation, as well as the enhancement of LR formation, which is through the activation of auxin biosynthesis and auxin signaling pathways.

Summary

How wounding triggers organ regeneration is among the most important questions in developmental biology. The induction of LRs upon wounding of the PR is generally observed, and has been extensively used in agricultural or horticultural techniques such as root pruning (Schupp and Ferree 1987), however, it has not been the subject of molecular genetic studies. In the present study, genetic, pharmacological, and molecular biology analyses were empolyed to reveal how LR formation were regulated by the wounding of the PR, both by PR cutting (Chapter 1) or by treatment of the damage associated molecular pattern *At*Peps to the tip of the PR (Chapter 2).

The root system is essential for the growth and development of the plants, as it is capable of anchoring to the ground, and absorbing water, minerals, and nutrients (Bellini et al. 2014). As the plants are sessile, the ability to response to adverse growth conditions, namely the plasticity of the RSA, is critical for the fitness and survival of the plants. With different soil moisture content, nutrient distribution, or various biotic or abiotic damages, the RSA adjust its morphology to gain fitness to the ever changing environment (Van Norman et al. 2013).

During plant growth, the PR and LRs coordinate to build an efficient RSA. When the PR is injured, the plants regenerate more LRs to compensate the defect of the RSA. This represents an important aspect of plants' high plasticity and adaptability. However, the underlying mechanisms are rarely studied. I aim to elucidate how plants regenerate the LRs when the PR is damaged, to maintain a balanced growth of the RSA.

In this study, either removal of the PR tip by root pruning or inhibition of PR elongation by application of *At*Pep1 and *At*Pep2 to the root tip promoted LR regeneration with an increase of LR density and LR length.

I showed in Chapter 1 that with root pruning, local auxin biosynthesis occurred and the auxin accumulated near the cut end, which promotes the new initiation of LRs proximal to the cut end. *YUC9* and other redundant *YUC* family genes are responsible for the elevation of auxin level after root cutting. Coordinated with the enhanced PAT activity, auxin biosynthesis triggered the auxin accumulation and subsequent activation of auxin signaling and LR formation pathways.

As showin in Chapter 2, AtPep1 and AtPep2 treatment on root tip induced the ectopic expression of the receptors PEPR1 and PEPR2 in the root tips, where PEPR2 is mainly responsible for the perception of the AtPeps and activated the auxin signaling pathway, which is marked by the enhanced expression level of pIAA19.:GUS in the root tip and elongation zone. The inhibition of PR elongation by AtPep1 and AtPep2 may be through the disturbance of cell cycle in root tip, where pCYCLIN B.:GUS expression was enhanced. Plants exposed to the cell cycle inhibitor in root tip showed similar root morphology changes as plants with AtPep1 or AtPep2 treatment. AtPeps' effect on root morphology change is independent of their role on pattern-triggered immunity, as flg22 and elf18 were unable to trigger the same response in the root.

Mechanical stresses like press, cut at meristem, or cut at elongation zone induced the ectopic expression of *PEPR1* and *PEPR2* in root tip. Salt stress though did not triggered the ectopic expression, enhanced the expression of *PEPR1* and *PEPR2* in the mature zone. The induction of PEPR receptors by the abiotic environmental stresses suggests the role of *At*Peps in response to external stimulus. What is the underlying biological meaning of PEPRs' induction by abiotic stresses remained to be investigated. Root pruning and *At*Peps treatment represent two distinct types of environmental stress with the common effect of PR elongation inhibition and LRs formation promotion. Their effects though perceived through different upstream cassettes, led to the same outcome of root morphology changes, which is regulated by the common integrator auxin.

These data revealed how plants coordinate the growth of PR and LRs in response to different environmental signals, through the activation of auxin signaling pathway. The upstream factors that conntect the wounding signaling and auxin signailing is still a missing link between the environmental stimuli and plant development. How the suppression of root apical meristem activity lead to the promotion of auxin signaling and LR formation also remained to be understand. These two questions raised by the present study will my future interest.

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Tables

Genotype	Intact plants	Root-cut plants
wild-type (WT)	4.7±0.3	7.0±0.3
msg2-1	1.1±0.3	7.5±0.3
shy2	0.0±0.0	16.2±4.4
slr	0.0±0.0	0.0±0.0
crane	0.5 ± 0.2	4.7±0.5
tir1-1 afb2-3	2.2±0.2	4.3±0.3
arf7 arf19	0.0±0.0	4.5 ± 2.4
arf6 arf8	1.1±0.3	8.7 ± 0.5

Table 1.1 LR number in auxin signaling mutants

LR number within the 12 mm area from the root-shoot junction (RSJ) was counted. Results were indicated by mean \pm SE, n = 10.

Gene name	Primer name	Primer Sequence (5'-3')	Reference
ACTIN2	ACTIN2-27S	5'-CGCTCTTTCTTTCCAAGCTCATA-3'	Muto et al. 2007
	ACTIN2+55AS	5'-CCATACCGGTACCATTGTCACA-3'	
IAA19	IAA19+390S	5'-CTTCGGTTTCCGTGGCATCG-3'	This study
	IAA19+521AS	5'-CATGACTCTAGAAACATCCC-3'	
ARF19	ARF19+2988S	5'-ACAGCTCGAAGATCCGCTAACC-3'	This study
	ARF19+3098AS	5'-TGCACGCAGTTCACAAACTCTTC-3'	
LBD16	LBD16+197S	5'-TCCATGATCGATGTGAAGCTGTCG-3'	This study
	LBD16+323AS	5'-TGTGATTGCAAGAAAGCCACCTG-3'	
LBD18	LBD18+274S	5'-TCCGATGCTGTCGTAACAATTTGC-3'	This study
	LBD18+390AS	5'-TTCTGCCTGTAGATTCACCACCTG-3'	
LBD29	LBD29+274S	5'-GCAAAAATCATGCTTTGTGCTGCT-3'	Blacha 2009
	LBD29+356AS	5'-TTTGCTCTCCAACAACAGGTTGTG-3'	
PIN1	PIN1+1546S	5'-GGCATGGCTATGTTCAGTCTTGGG-3'	This study
	PIN1+1661AS	5'-ACGGCAGGTCCAACGACAAATC-3'	
PIN3	PIN3+1154S	5'-AAGGCGGAAGATCTGACCAAGG-3'	This study
	PIN3+1248AS	5'-TGCTGGATGAGCTACAGCTTTG-3'	
PIN7	PIN7+1699S	5'-CGTGTGGCCATTGTTCAAGCTG-3'	This study
	PIN7+1794AS	5'-CCCTGTACTCAAGATTGCGGGATG-3'	
YUC9	YUC9+542S	5'-ATAAGTCCGGCGAGAAATTCAGAG-3'	This study
	YUC9+682AS	5'-TCGGTAAAACATGAACCGAG-3'	
TAA1/SAV3	TAA1+67S	5'-TTCGTGGTCAATCTGGATCATGG-3'	This study
	TAA1+156AS	5'-ACCACGTATCGTCACCGTACAC-3'	

Table 1.2 Gene-specific primers used for qRT-PCR analysis

Figures



Figure 1.1 Root-cutting induced increase in lateral root (LR) number (RCN) and root-cutting induced increase in LR growth (RCG) in wild-type (WT) and *massugu2/indole-3-acetic acid19* (*msg2-1*) plants.

(A) Four-day-old plants were transferred to new medium and incubated for 1 d before root cutting. Photographs were taken 4 d after root cutting. Scale bars = 1 cm. Red arrowheads indicate the 12 mm point from RSJ that corresponds to the cut point. (B) The number of LRs was counted in the 12 mm area from the RSJ. (C) Growth rate of the first emerged LR. (D) Total length of the root system of intact or root-cut plants 4 d after root cutting. (E) Progress of LR development after root cutting. The roots of 5-d-old plants were cut at 0 h. The number of lateral root primordia (LRP) or LRs was counted at the indicated time points and the sum of LRP and LR number was indicated as total. (F) LR number within 0–6mm or 6–12mm of the cut end. Error bars indicate the SE (n=16). *Significant differences between root-cut and intact plants (Student's *t* test, *P*<0.05).



Figure. 1.2 Root cutting induced LR formation in auxin-related mutants.

Four-day-old plants were transferred to new medium and incubated for 1 d before root cutting. After root cutting, plants were incubated at control temperature $(23^{\circ}C)$ (A-F) or high temperature $(28^{\circ}C)$ (G, H). Photographs were taken 4 d (A-C, E, G) or 16 d (F, H) after root cutting (DAC). Scale bars = 1 cm. Red arrowheads indicate the 12 mm point from root-shoot junction that corresponds to the cut point. (D) The number of LRs was counted in the 12 mm area from the root-shoot junction. Error bars indicate the SE (n=16). *Significant differences between root-cut and intact plants (Student's *t* test, *P*<0.05).



Figure 1.3 Lateral root primordia (LRP) and lateral root (LR) number near the cut end.

Four-day-old plants were transferred to new medium and incubated for 1 d before root cutting. The roots of 5-d-old plants were cut at 0 h. The number of LRP or LRs was counted within the 1.24 mm area from the cut end and the sum of LRP and LR number was indicated as total. Error bars indicate SE (n = 16). *Significant differences compared with intact plants (Student's *t* test, *P*<0.001).



Figure 1.4 Auxin-signaling genes are induced by root cutting.

(A–C) Relative expression of *IAA19*, *ARF19*, and *LBD29* after root cutting. Error bars indicate the SE of three independent biological replicates. (D) The expression pattern of *IAA19*. The roots of six-day-old seedlings expressing *pIAA19*. GUS were cut at 12 mm from RSJ for 6 hours. β -glucuronidase (GUS) staining was observed in the cut end of root-cut plants and corresponding area in intact plants. Scale bar = 0.1 mm.



Figure 1.5 Changes of the relative expression level of LR formation-related genes (*LBD16* and *LBD18*), auxin transport genes (*PIN1*, *PIN3*, and *PIN7*), and auxin biosynthesis gene (*TAA1/SAV3*) in response to root cutting.

(A–F) RNA was extracted from 0–11 mm from the cut end. (G) RNA was extracted from 0–2.5 mm from thecut end. Error bars indicate SE from three independent biological replicates.



Figure 1.6 RCN is robust to auxin transport inhibitors but not to auxin biosynthesis inhibitors.

Four-day-old plants were transferred to medium with or without auxin transport (NPA) N-1-naph-thylphthalamic (A), inhibitors acid 2-[4-(diethylamino)-2-hydroxybenzoyl] (BUM) (B), benzoic acid or 2,3,5-triiodobenzoic acid (TIBA) (C) or the auxin biosynthesis inhibitor L-Kynurenine (D) and incubated for 1 d before root cutting. The number of LRs was counted in the 12 mm area from the RSJ 4 d after root cutting. (E) WT and sav3-2 plants were treated with difference concentrations of yucasin. (F) The combination of yucasin and NPA abolished LR formation in both intact and cut plants. Error bars indicate SE (n = 16). *Significant differences in root-cut versus intact plants (A–E) and treated versus control plants (F) (Student's t test, P < 0.05).



Figure 1.7 IAA is induced by root cutting.

Auxin concentration was measured after root cutting at the indicated time points. Error bars indicate the SE of three independent biological replicates. *Significant differences compared with 0 h (Student's t test, P < 0.05).



Figure 1.8 LR number of WT and *msg2-1* plants was induced by 1-naphthaleneacetic acid (NAA).

(A) Four-day-old plants were transferred to medium with or without NAA and incubated for 1 d before root cutting. Photographs were taken 4 d after root cutting. Scale bars = 1 cm. Red arrowheads indicate the point 12 mm from the RSJ where the root was cut. (B) The number of LRs was counted within the 12 mm area from the RSJ 4 d after root cutting. Error bars indicate the SE (n=20). *Significant differences compared with plants in control medium (Student's *t* test, P<0.05).





Four-day-old plants were transferred to medium without (A) or with (B, C) N-1-naph-thylphthalamic acid (NPA) and incubated for 1 d before root cutting. The number of LRs was counted within the 12 mm area from the RSJ 4 d after root cutting. Error bars indicate the SE (n=16). *Significant differences compared with WT (Student's t test, P<0.01). (C) Plants were in Landsberg*erecta* background.



Figure 1.10 The role of *YUCCA9* (*YUC9*) on root cutting.

Four-day-old WT (A) and yuc9 (B) plants were transferred to medium with or without NPA and incubated for 1 d before root cutting. Photographs were taken 4 d after root cutting. Scale bars = 1 cm. Red arrowheads indicate the 12 mm point from RSJ that corresponds to the cut point. (C) LR number of WT and yuc9following root cutting in the presence or absence of NPA. LR number was counted within 12 mm from the RSJ 4 d after root cutting. (D) Progress of LR development after root cutting. The roots of 5-d-old plants were cut at 0 h. The number of LRP or LRs was counted and the sum of LRP and LR number was indicated as total. (E) Relative expression level of YUC9 after root cutting. (F) IAA level of WT and yuc9 at the indicated time points. Error bars indicate SE from 16 seedlings (C, D) or from three independent biological replicates (E, F). *Significant differences compared with WT plants (C, D) or 0 h (F) (Student's t test, P<0.05).



Figure 1.11 Jasmonic acid (JA) induced LR formation in WT but not in *yuc9*. (A) Four-day-old plants were transferred to medium with or without JA and incubated for 1 d before root cutting. The number of LRs was counted in the 12 mm area from the RSJ 4 d after root cutting. JA (B) and JA-Ile (C) concentration were measured after *At*Pep1 treatment at the indicated time points. Error bars indicate the SE (n=16) (A) or (n=3) (B, C). *Significant differences compared with plants in control medium (Student's *t* test, *P*<0.05).



Figure 1.12 Functional redundancy of *YUC* family genes in RCN.

(A) Increases in LR number under different concentration of yucasin in the presence of NPA. Increases in the LR number were calculated by subtracting the LR number of intact plants from root-cut plants. (B) Four-day-old plants were transferred to medium with or without NPA and incubated for 1 d before root cutting. Photographs were taken 4 d after root cutting. Scale bars = 1 cm. Red arrowheads indicate the 12 mm point from RSJ that corresponds to the cut point. LR number of plants after root cutting in the absence (C) or presence (D) of NPA. LR number was counted within 12 mm from the RSJ 4 d after root cutting. Scale bars = 1 cm. Scale bars = 1 cm. Error bars indicate SE (n=16). *Significant differences compared with WT (Student's *t* test, *P*<0.01).



Figure 1.13 Synergysticeffect of auxin biosynthesis and polar auxin transport (PAT) on RCN.

(A) The number of LRs was examined in PAT related mutants with or without yucasin treatment. Reduction of LR number in different concentration of auxin transport inhibitors NPA (B) and TIBA (C). The number of LRs was counted in the 12 mm area from the RSJ 4 d after root cutting. Error bars indicate SE (n = 16). *Significant differences compared with plants in control medium (Student's *t* test, P<0.05).



Figure 1.14 Model of the synergistic regulation of RCN by auxin biosynthesis and polar auxin transport.

Root cutting activates the expression of *YUC9* and other *YUC* family genes, resulting in the elevation of auxin level, which further induces PAT-related gene expression. Enhanced PAT activity leads to auxin accumulation and activation of downstream auxin signaling pathways which induces LR initiation and LR development.



Figure 2.1 AtPep1 induced LR formation dose dependently.

Four-day-old plants were transferred to new medium without (A–C, F) or with (D, E) NPA and incubated for 1 d before AtPep1 treatment. 4 d after treatment, plants were photographed (A) and LR density (B, D, G), PR length (C, E), and PR growth rate (F) were measured. (G) Four-day-old plants were transferred to new medium, LR density was measured 5 d later. Scale bar = 1 cm. Error bars indicate the SE (n = 16). *Significant differences compared with control treatment (A, B) or WT plants (C) (Student's *t* test, *P*<0.05).



Figure 2.2 *At*Pep1-induced LR formation is mediated by PEPR2.

Four-day-old plants were transferred to new medium without (A–D) or with (E, F) 1 μ M NPA and incubated for 1 d before *At*Pep1 treatment (1 μ M). 4d after treatment, photographs were taken (A, B), and LR density (C, E) or PR length (D, F) were measured. Scale bar = 1 cm. Error bars indicate the SE (n = 16). *Significant differences compared with WT plants (Student's *t* test, *P*<0.001).



Figure 2.3 Functional similarities between *At*Pep1 and *At*Pep2.

(A) Four-day-old plants were transferred to new medium and incubated for 1 d before being treated with different AtPeps (1 μ M). LR density (A, C) or PR length (B, D) were measured 4 d after treatment. Error bars indicate the SE (n = 16). *Significant differences compared with control treatment (A, B) or WT plants (C, D) (Student's *t* test, *P*<0.001).



Figure 2.4 *At*Pep1-induced LR formation is mediated by auxin signaling.

(A) The expression pattern of *IAA19*. The roots of 5-day-old seedlings expressing pIAA19::GUS were treated with 1 mM AtPep1 for 6 hours and GUS staining was observed. (B–F) Four-day-old plants were transferred to new medium without (B, C) or with 1 mM NPA (D, E) or 50 mM yucasin (F) and incubated for 1 d before AtPep1 treatment. 4 d after treatment photographs were taken (B) and LR density was measured (C–F). (G) Auxin concentration was measured after AtPep1 treatment at the indicated time points. Scale bars = 0.1 mm (A) or 1 cm (B). Error bars indicate the SE of three independent biological replicates. Error bars indicate the SE (n = 16) (C–F) or (n=3) (G). * Significant differences compared with control treatment (B, E) or WT plants (C, D) (Student's t test, P<0.01).



Figure 2.5 Involvement of JA signaling in *At*Pep1-induced LR formation.

Four-day-old plants were transferred to new medium with 1 μ M NPA and incubated for 1 d before *At*Pep1 treatment (1 μ M). LR density was measured 4 d after treatment. Error bars indicate the SE (n = 16). * Significant differences compared with WT plants (C, D) (Student's *t* test, *P*<0.05).



Figure 2.6 PR inhibition and LR induction are two separate processes.

Four-day-old plants were transferred to new medium with 1 μ M NPA and incubated for 1 d before *At*Pep1 treatment (1 μ M). LR density was measured 4 d after treatment. Error bars indicate the SE (n = 16). * Significant differences compared with control treatment (Student's *t* test, *P*<0.05).



Figure 2.7 Root tip is the target tissue of *At*Pep1.

Four-day-old plants were transferred to new medium and incubated for 1 d before AtPep1 treatment (1 μ M). 4 d later photograph were taken (A) and LR density (B), PR length (C) and 1st LR length (D) were measured. Arrowheads indicate 0 and 12 mm from RSJ. Scale bars = 1 cm. Error bars indicate the SE (n = 16). * Significant differences compared with control treatment (Student's *t* test, *P*<0.05).



Figure 2.8 *At*Pep1 induces ectopic expression of PEPR1 and PEPR2 in root tip. The expression pattern of *PEPR1* (A, C) and *PEPR2* (B, D) with *At*Pep1 treatment in root tip (1 μ M) (A, B) or 0–12 mm from RSJ (C, D). The roots of 5-day-old seedlings expressing *pPEPR1::GUS* or *pPEPR2::GUS* were treated with *At*Pep1 for 4.5 h and GUS staining was observed. Scale bars = 0.1 mm.



Figure 2.9 Disturbance of cell cycle in root tip suppresses PR growth and promotes LR formation.

The expression pattern of *CYCLIN B* in root tip with *At*Pep1 (A) or oryzalin (B) treatment. The roots of 5-day-old seedlings expressing *p CYCLIN B* were treated with 1 μ M Atpep1 or 100 μ M oryzalin for 4.5 h and GUS staining was observed. Scale bars = 0.1 mm. (C) Four-day-old plants were transferred to new medium and incubated for 1 d before oryzalin treatment on root tip. Photographs were taken 4 d after treatment. Scale bars = 1 cm. LR density (D) and PR length (E) were measured 4 d after treatment. Error bars indicate the SE (n = 16). * Significant differences compared with control treatment (Student's *t* test, *P*<0.05).



Figure 2.10 Perception of AtPep1 but not of the MAMPs flg22 or elf18 affected root growth.

(A) Four-day-old plants were transferred to new medium and incubated for 1 d before being treated with 1µM AtPep1, flg22, or elf18 on root tip. Photographs were taken 4 d after treatment. Scale bars = 1 cm. LR density (B) and PR length (C) were measured 4 d after treatment. Error bars indicate the SE (n = 8). * Significant differences compared with control treatment (Student's t test, P < 0.001).



Figure 2.11 PEPR1 and PEPR2 expression was induced by different abiotic stresses.

The roots of 5-day-old seedlings expressing pPEPR1::GUS (A, C, E, G) and pPEPR2::GUS (B, D, F, H) were treated with different abiotic stress: apply 300 mM NaCl in root tip (A, B), press root tip with tweezer (C, D), cut root at meristem (E, F), or cut root at elongation zone (G, H). GUS staining was performed 4 h after treatment. Scale bars = 0.1 mm.



Figure 2.12 *per1-1 pepr2-1* was more sensitive to salt treatment.

Four-day-old plants were transferred to new medium with 100 mM NaCl and incubated for 4 d before observation. Plants with yellow or green cotyledon were counted.



Figure 2.13 Model of *At*Pep1 and *At*Pep2-induced inhibition of PR elongation and promotion of LR formation.

In the root tip, *At*Pep1 and *At*Pep2 are perceived by PEPR2 and enhance the ectopic expression of PEPR2 in root tip, which further disturbs the normal cell cycle marked by the induction of *CYCLIN B* expression. This results in the inhibition of PR elongation, as well as the enhancement of LR formation through the activation of auxin biosynthesis and auxin signaling pathways.