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Transcriptional regulation of auxin metabolic-enzyme genes during tomato fruit development

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ABSTRACT

Auxin plays crucial roles in many stages of fruit development. To understand the mechanism of regulation exerted by auxin during fruit development, we analyzed the variations in the levels of indole-3-acetic acid (IAA) and its precursors, as well as the variations in the expression of the genes involved in IAA metabolism in developing tomato (*Solanum lycopersicum* 'Ailsa Craig') fruits. The concentrations of IAA and its precursor indole-3-pyruvic acid (IPyA) increased in the ovary three days after anthesis. The transcript levels of the auxin biosynthetic-enzyme genes *SlTAR2*, *ToFZY1*, *ToFZY2,* and *ToFZY5* also increased during this stage. These results suggest that the *de novo* IAA biosynthesis in the pollinated ovaries is controlled through the IPyA pathway, and is involved in fruit set and early fruit growth. Contrarily, the transcripts of the auxin inactivating-enzyme genes *SlGH3-9* and *SlGH3-15* were accumulated in the unpollinated ovaries and decreased after pollination. This suggests that the auxin inactivating-enzymes might be involved in the control of IAA levels in the unpollinated ovaries. Further, we found that application of synthetic auxin 2,4-dichlorophenoxyacetic acid on the unpollinated ovaries increased the transcript levels of *SlTAR2*, *ToFZY1*, *ToFZY2,* and *ToFZY5* and decreased those of *SlGH3-9* and *SlGH3-15*. These results suggest that elevated auxin signaling via pollination or exogenous auxin treatment might promote further auxin accumulation in the ovaries.

1. Introduction

Fruit development is a complex and tightly regulated process. In the case of tomato (*Solanum lycopersicum*), which is the model species of fleshy fruit plants, this process can be divided into several phases. These include ovary development, pollination/fertilization and subsequent fruit set, cell division (that continues for seven to ten days after pollination), cell expansion (that occurs after cell division and depends on genotype), and ripening (Gillaspy et al., 1993). Auxin has important roles in many stages of fruit development (Gillaspy et al., 1993; Pattison et al., 2014). Exogenous applications of synthetic auxins, such as 4-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid (2,4-D), to the ovary can induce fruit set and development without pollination/fertilization in several plant species (Abad and Monteiro, 1989; Nothmann et al., 1983). Manipulation of the auxin biosynthetic-enzyme gene in ovaries can also induce parthenocarpic fruit development in eggplant and tomato (Ficcadenti et al., 1999; Rotino et al., 1997). These results indicate that the appropriate control of endogenous auxin content within the ovary is crucial for fruit set and development. However, the molecular mechanisms underlying auxin regulation are not fully understood in fruit crops.

Indole-3-acetic acid (IAA) is the major naturally occurring active auxin in many plants. The tryptophan (Trp)-dependent and the Trp-independent routes have been proposed for IAA biosynthesis in plants (Normanly et al., 1993), although the first is believed to be the main one. This route includes several pathways, such as the indole-3-pyruvic acid (IPyA), tryptamine (TRA), indole-3-acetamide (IAM), and indole-3-acetaldoxime (IAOx) pathways (Fig. S1; Ljung, 2013). The IPyA pathway is currently considered the major IAA biosynthetic pathway in plants (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al.,

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BR, breaker; DAA, days after anthesis; DAO, DIOXYGENASE FOR AUXIN OXIDATION; FB, flower bud; Fl, flower; GH3, GRETCHEN HAGEN 3; GUS, β-glucuronidase; HPLC/MS/MS, high performance liquid chromatography coupled with a tandem quadrupole mass spectrometer; IAA, indole-3-acetic acid; IAM, indole-3-acetamide; IPyA, indole-3-pyruvic acid; IPyA-TAZ, IPyA-thiazolidine; ML, mature leaf; MRM, multiple reaction monitoring; MS, mature stem; Ov, ovary; oxIAA, 2-oxindole-3-acetic acid; Pe, petal; PI, pink; Pol, pollinated ovary; R, root; RR, red-ripe; Se, sepal; St, stamen; TAA1/TARs, tryptophan aminotransferases; TAM, tryptamine; Trp, tryptophan; TU, turning; YL, young leaf; YS, young stem

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2011). Here, tryptophan aminotransferases (TAA1/TARs) catalyze the conversion of Trp to IPyA, and the conversion of IPyA to IAA is catalyzed by the YUCCA (YUC) family of flavin-containing monooxygenases. The *YUC* and *TAA1/TAR* family genes are widely conserved across plant species, and mutant plants for these genes showed auxin-deficient phenotypes (Cheng et al., 2006; Stepanova et al., 2008; Tao et al., 2008; Tobeña-Santamaria et al., 2002). The IAM pathway is well known in IAA-synthesizing bacteria (Mano and Nemoto, 2012), and IAM has also been found as an endogenous compound in plants (Sugawara et al., 2009). Furthermore, the overexpression of the bacterial *iaaM* gene, which codes the tryptophan-2-monooxygenase that catalyzes the conversion of Trp to IAM, can induce the overproduction of IAA in several plants (Ficcadenti et al., 1999; Rotino et al., 1997). Thus, the IAM pathway is important route for IAA biosynthesis in plants. Although TRA has been identified in many plant species and proposed as the precursor of indole alkaloid, serotonin, and IAA (Mano and Nemoto, 2012; Quittenden et al., 2009), the pathway for IAA synthesis remains poorly understood. The IAOx pathway has been well studied in *Arabidopsis*. However, the *CYP79B* genes that are involved in a key step of this pathway have only been found in Brassicaceae (Sugawara et al., 2009). Thus, this pathway is considered species-specific.

The level of IAA is regulated by several metabolic pathways other than biosynthetic pathways. GRETCHEN HAGEN 3 (GH3) family proteins are known to be involved in the conjugation of amino acids to several plant hormones, and these family genes are classified into groups I, II, and III (Staswick et al., 2002). The expression of group II *GH3* genes was upregulated after an exogenous treatment with auxins, and proteins catalyzed the formation of IAA–amino acid conjugates (Staswick et al., 2005). Several of these IAA-amino acid conjugates are irreversible inactive forms, whereas others function as IAA-storage forms (Korasick et al., 2013). The oxidation of IAA to 2-oxindole-3-acetic acid (oxIAA) is another irreversible inactivation reaction of IAA in plants (Pěnčík et al., 2013). DIOXYGENASE FOR AUXIN OXIDATION (DAO) proteins, which convert IAA to oxIAA, were recently identified in rice and *Arabidopsis* (Mellor et al., 2016; Porco et al., 2016; Zhang et al., 2016a; Zhao et al., 2013). The GH3-dependent conjugation and DAO oxidation pathways are currently considered the main IAA inactivation pathways in plants. Other metabolic routes have also been proposed for the regulation of IAA in plants, but complete metabolic pathways remain unclear.

Recently, several studies have reported the expression of auxin biosynthetic-enzyme genes in developing tomato ovaries (Mariotti et al., 2011; Pattison et al., 2015; Zhang et al., 2016b). However, these reports lack detailed information regarding the relationship between IAA and related compound contents and the expression of auxin biosynthetic-enzyme genes. Moreover, the regulation of auxin inactivating-enzyme genes is poorly understood in tomato. This study aimed to elucidate the regulation of auxin metabolism in tomato fruit development. Specifically, we aimed to clarify which auxin biosynthesis and inactivation pathways have important roles in developing fruits. We first quantified the contents of endogenous IAA and its precursors during various developmental stages of the tomato fruits, and then examined the expression of auxin biosynthetic- and inactivating-enzyme genes during fruit development. We also analyzed the expression of the auxin metabolic-enzyme genes in response to exogenous auxin treatment.

2. Materials and methods

2.1. Plant materials and growth conditions

Tomato (*Solanum lycopersicum*) cultivar Ailsa Craig plants were grown on fertilized granulated soil (Nihon Hiryo Corporation, Tokyo, Japan), in a phytotron (Koito Electric Industries, Yokohama, Japan) under a 14:10 h light:dark cycle, at an irradiance of 160 μmol m^{-2} s^{-1}, under 25 °C (light period) or 20 °C (dark period). For the gene expression analysis, flowers were emasculated two days before anthesis (-2 DAA) to prevent self-pollination. Manual pollination was carried out at anthesis (0 DAA). For the auxin treatment, 20 ng of 2,4-D (Serrani et al., 2007; Wako Pure Chemical Industries, Osaka, Japan) was applied on the unpollinated ovaries in 20 μL of 10% ethanol, 0.1% Tween 20. Same volumes of the solvent solution were applied on the control ovaries. The ovaries were harvested three days after treatment and used for RNA extraction. We used at least 15 plants for the pollination experiment and at least four plants for the auxin application experiment. In each treatment, three biological replicates were used for gene expression analysis or quantification of IAA-related compounds. Two repeat experiments were performed in each case.

2.2. Quantification of IAA and its precursors

For analysis of IAA, Trp, TRA, and IAM, tomato whole ovaries or fruits (about 50 mg) were homogenized in liquid nitrogen and placed in 3 ml 80% acetone. The [$^{13}C_6$]IAA and [$^{2}H_5$]Trp (both obtained from Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA), and [$^{2}H_5$]IAM and [$^{2}H_4$]TRA (both from C/D/N Isotopes Inc., Quebec, Canada) added to the extracts served as internal standards. After overnight extraction at 4 °C, solids were separated by centrifugation and re-extracted for 30 min in 3 ml of the same extraction solution. The extract was evaporated and dissolved in 3 ml 1% acetic acid and purified by solid-phase extraction using the Oasis HLB column (60 mg; Waters, Milford, MA, USA) according to Novák et al. (2012). The eluate was evaporated, dissolved in 3 ml 1% acetic acid, and applied to an Oasis MCX column (60 mg, Waters) that had been conditioned with 3 ml 100% methanol and 3 ml water successively and equilibrated with 1% acetic acid. After washing the column with 3 ml 2% formic acid, IAA and IAM were eluted with 6 ml 100% methanol, and then Trp and TRA were eluted with 6 ml methanol containing 5% ammonia. Each fraction was evaporated, and the fraction containing Trp and TRA was dissolved in water/methanol/acetic acid (89/10/0.1, v/v/v) and analyzed by high performance liquid chromatography coupled with a tandem quadrupole mass spectrometer using an electrospray interface (HPLC/MS/MS; 1260 Infinity LC System equipped with a 6460 Triple Quadrupole LC/MS system; Agilent Technologies, Santa Clara, CA, USA). The fraction containing IAA and IAM was dissolved in 3 ml 1% acetic acid and applied to an Oasis WAX column (60 mg, Waters) that had been conditioned with 3 ml 100% methanol and 3 ml water successively and equilibrated with 1% acetic acid. After washing the column with 3 ml 1% acetic acid, IAM was eluted with 3 ml methanol and IAA was eluted with 6 ml 80% methanol containing 1% acetic acid. The eluate was evaporated, dissolved in water/methanol/acetic acid (89/10/0.1, v/v/v) and analyzed by HPLC/MS/MS. For the analysis of IPyA, [$^{13}C_{11}$,$^{15}N_1$]IPyA was synthesized from [$^{13}C_{11}$,$^{15}N_2$]Trp (Cambridge Isotope Laboratories) according to the method of Liu et al. (2012) and used as an internal standard. Extraction and derivatization were carried out according to the method of Novák et al. (2012) with slight modifications. Tomato whole ovaries or fruits (about 50 mg) were homogenized in liquid nitrogen and extracted in 3 ml cold sodium phosphate buffer (50 mM, pH 7.0) containing 1% diethyldithiocarbamic acid sodium salt. Extraction was carried out at 4 °C with continuous shaking for 15 min and then centrifuged at 4 °C. For derivatization, 6 ml 0.25 M cysteamine solution (pH 8.0) was added to 1 ml extract and incubated for 1 h at room temperature. After derivatization to IPyA-thiazolidine (IPyA-TAZ), the sample was adjusted to pH 2.7 using 1 M HCl, and passed through an Oasis HLB column (60 mg, Waters) that had been conditioned with 3 ml 100% methanol and 3 ml water successively and equilibrated with sodium phosphate buffer (50 mM, pH 2.7). After washing the column with 6 ml 5% methanol, IPyA-TAZ was eluted with 6 ml 80% methanol. This eluate was then evaporated and dissolved in water/methanol/acetic acid (79.95/20/0.05, v/v/v) and analyzed in the HPLC/MS/MS system. The quantification of compounds was