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Original Paper

Altered levels of primary metabolites in response to exogenous indole-3-acetic acid in wild type and auxin signaling mutants of *Arabidopsis thaliana*: A capillary electrophoresis-mass spectrometry analysis

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Abstract A comprehensive analysis of the levels of primary metabolites in wild type (WT) and several auxin-signaling mutants namely, *tir1*, *slr* and *arf7 arf19* of *Arabidopsis thaliana* has been performed using CE-MS, a technique particularly sensitive for the measurement of polar compounds. We first measured the levels of primary metabolites in shoots and roots, most of the analyzed metabolites were found to be quantitatively and qualitatively comparable in WT and three kinds of mutants (*tir1*, *slr* and *arf7 arf19*). Some amino acids such as GABA, Arg, Orn, Val, Thr, Leu and Ile exhibited a unique pattern of distribution between shoots and roots in both WT and the mutants. On the other hand, the mutant *slr* showed a quite different pattern of metabolites measured in the present study.

Subsequently, the responses of primary metabolites to a short-term (60 min) application of exogenous IAA (10^{-7}, 10^{-8} M) in WT and the mutants were characterized. Due to IAA treatments, some amino acids such as GABA in WT roots and Gly and Ala in WT shoots were altered, but not in the mutants. Gln was altered in *slr* shoots by 10^{-7} M IAA treatment. Levels of G6P from the glycolic pathway were altered in WT roots and those of 2PG, 3PG were altered in *tir1* shoots in response to IAA treatments. The levels of succinate in TCA cycle were altered by IAA treatments in WT shoots but not in the mutants. IAA treatment inhibited the respiration in WT roots. The suppression of respiration might account for the IAA-dependent alteration of some metabolites. Difference of auxin responses between WT and auxin-signaling mutants suggests that some metabolic processes are under IAA control.

Key words: *Arabidopsis*, auxin, auxin signaling mutants, CE-MS, metabolome, metabolomics.

Indole-3-acetic acid (IAA), the predominant naturally occurring auxin, regulates many morphological and physiological phenomena in plants including cell division, cell elongation, various developmental processes, vascular differentiation, apical dominance and tropic responses to light and gravity (Davies 2010; Vanneste and Friml 2009; Woodward and Bartel 2005). Recent studies involving diverse molecular and genetic approaches have led to a clearer understanding of the auxin biosynthetic pathway, the regulation of auxin transport, the regulation of auxin-mediated gene expression and the components of auxin signaling in plants (Hayashi 2012; Mashiguchi et al. 2011). In response to the exogenously applied plant hormones, significant changes in the gene expression occur that are followed by corresponding changes in the levels of proteins. There is, however, a dearth of information concerning the hormone induced changes in the levels of specific metabolites. Obviously, such information is necessary to establish a link between specific low

Abbreviations: 2OG, 2-oxoglutaric acid; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconic acid; Ala, L-alanine; ARF, AUXIN RESPONSE FACTOR; Arg, L-arginine; Asn, L-asparagine; Asp, L-aspartic acid; AST, asparatate aminotransferase; C10F, camphor-10-sulfonic acid; CE-MS, capillary electrophoresis-mass spectrometry; Cys, L-cysteine; DAG, days after germination; DHAP, dihydroxyacetone phosphate; F6P, fructose-6-phosphate; FBP, fructose-1, 6-bisphosphate; FW, fresh weight; G1P, glucose-1-phosphate; G3P, glycerol-3-phosphate; G6P, glucose-6-phosphate; GABA, γ-aminobutyric acid; GAP, glyceraldehyde-3-phosphate; GC-MS, gas chromatography-mass spectrometry; Gln, L-glutamine; Glu, L-glutamic acid; Gly, glycine; His, L-histidine; IAA, indole-3-acetic acid; Ile, L-isoleucine; LC-MS, liquid chromatography-mass spectrometry; Leu, L-leucine; Lys, L-lysine; MeS, L-methionine sulfone; Met, L-methionine; Orn, L-ornithine; PEP, phosphoenolpyruvate; Phe, L-phenylalanine; Pro, L-proline; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; Ser, L-serine; Thr, L-threonine; Trp, L-tryptophan; Tyr, L-tyrosine; Val, L-valine; WT, wild type.

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molecular weight metabolites and hormone-regulated processes (Davies 2010). Indeed, the plant primary metabolic responses to IAA have been studied less since 1930s (Bonner 1936; Hackett and Thimann 1952; Marrè and Arrigoni 1957). Although it is known that the auxin and ethylene regulate flavonol biosynthesis through auxin signaling networks as reported in Lewis et al. (2011), there was almost no report about relationship between primary metabolites and auxin. It should be rewarding to address this issue with currently available genetic tools including specific mutants and sophisticated techniques allowing precise quantitative and qualitative analysis of diverse categories of plant metabolites.

Concerning the auxin signaling, nuclear auxin receptors, TRANSPORT INHIBITOR RESPONSE1 (TIR1) and TIR1-related F-box proteins (AFBs), are known to interact with AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins in the presence of auxin and promote their degradation through the ubiquitination by the $SCF^{TIR1/AFB}$ receptor complex (Guilfoyle and Hagen 2007; Kepinski and Leyser 2004). The *Aux*/*IAA*s are the auxin-inducible genes that encode short-lived nuclear proteins that repress auxin-responsive gene expression. In *Arabidopsis thaliana*, the *Aux*/*IAA* family is comprised of 29 members (Remington et al. 2004). The Aux/IAA protein heterodimerizes with AUXIN RESPONSE FACTOR (ARF) that is a transcription factor, thereby blocking the ARF-dependent transcriptional regulation of auxin-responsive genes. ARF family proteins (23 members in *A. thaliana*) bind to the Auxin-Responsive *cis*-Elements in the promoters/regulatory regions of the auxin-responsive genes, thereby activating or repressing their transcription. Therefore, the intracellular level of Aux/IAA repressors determines the activity of ARF transcriptional factor (Liscum and Reed 2002; Mockaitis and Estelle 2008).

In *A. thaliana*, many auxin response mutants have been characterized to elucidate the auxin signaling pathways in plants. The *transport inhibitor response1-1* (*tir1*) is a loss-of-function mutant of TIR1, which binds to auxin and interacts with Aux/IAA in the presence of auxin. The *tir1* mutant shows relatively weak auxin insensitivity including auxin-dependent hypocotyl elongation and auxin-inhibited root elongation (Ruegger et al. 1998). Because there exist the other nuclear auxin receptors, TIR1-related F-BOX proteins, AFBs, TIR1 contributes to auxin signaling with these AFBs (Dharmasiri et al. 2005). The dominant *solitary-root-1* (*slr1-1*) is a gain-of-function mutant in IAA14. The *slr* mutation completely blocks the lateral root formation, and also inhibits the root hair formation and the gravitropic responses of roots and hypocotyls (Fukaki et al. 2002). The auxin-signaling mutant *arf7 arf19*, a double mutant, also shows the defect in auxin-regulated growth and developmental processes. This has only few lateral root formation, reduced gravitropic responses of roots and hypocotyls, and impaired auxin-induced gene expression (Okushima et al. 2005; Wilmoth et al. 2005). Previous studies on the genetic and physical interactions between SLR/IAA14 and ARF7/19 suggest that the SLR/IAA14-ARF7-ARF19-mediated auxin signaling is involved in the auxin-regulated growth and developmental processes including lateral root formation (Fukaki et al. 2005). In our study, these three auxin-signaling mutants (*tir1*, *slr* and *arf7 arf19*) with no extreme morphological change were chosen to examine the changes in the levels of metabolites in response to the exogenous IAA.

In the last decade, as a consequence of technological advancements, it has been possible to precisely identify and quantify the metabolites employing mass spectrometry combined with gas chromatography (GC-MS) or liquid chromatography (LC-MS) in plant tissues, cells and organelles. The capillary electrophoresis-mass spectrometry (CE-MS) offers a tool particularly suitable and applicable for the analysis of polar metabolic compounds in plant tissues.

The metabolomic techniques have been successfully used in the medical field to find e.g. the biomarkers of various diseases over the past few years (Soga et al. 2006; Wang et al. 2011). The use of these techniques in the context of plants is associated with some difficulties in view of the plants possessing a greater number of metabolites as compared to the animal systems and the widely ranging concentrations (pico-to-micromolar) of the metabolites. Plant metabolomics is extensively developing as it is expected to contribute substantially to the functional elucidation of unknown genes and to the understanding of regulation and significance of metabolic processes in response to hormonal and environmental cues (Fiehn 2002; Kusano et al. 2011; Matsuda et al. 2012).

With the aim of getting insight into the role of IAA in the regulation of primary metabolism, the responses of specific (primary) metabolites in *A. thaliana* (WT and three auxin-signaling mutants namely, *tir1*, *slr* and *arf7 arf19*) to a short-term application of exogenous IAA have been characterized using CE-MS. Exogenous IAA rapidly induced some changes in the levels of certain specific primary metabolites in WT but not in the mutants. The significance of findings for auxin-dependent regulation of gene expression would be discussed.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana accession Columbia was used in this study. The *tir1* mutant was obtained from the Arabidopsis Biological Stock Center. The *slr* and *arf7-1 arf19-1* mutants have been described previously (Fukaki et al. 2002; Okushima et al. 2005;

Okushima et al. 2007).

After being sterilized for 15 min with 95% ethanol, seeds were sown on a gellan gum plate (0.6% (w/v)) containing Murashige and Skoog (MS) medium with a composition of 20.6 mM NH_4NO_3, 18.8 mM KNO_3, 1.25 mM KH_2PO_4, 0.1 mM H_3BO_3, 0.1 mM $MnSO_4$, 0.037 mM $ZnSO_4$, 1 μM KI, 1 μM Na_2MoO_4, 0.1 μM $CuSO_4$, 0.1 μM $CoCl_2$, 3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 0.1 mM FeNa-EDTA, 1% Sucrose, 0.01% myo-inositol, 0.3 mg ml^{-1} thiamine, 0.5 mg l^{-1} nicotinic acid, 0.05 mg l^{-1} pyridoxine-HCl. The pH of the MS medium was adjusted to 6.2 with KOH. After sowing, the plates were kept at 4°C in the dark for over 36 h. Then, the plates were transferred from 4°C to an incubator (BIOTRON, NK system, JAPAN) at 22.5 to 23.5°C under continuous light (270–340 μmol m^{-2} s^{-1}), and that day was defined as DAG (days after germination) 0.

In order to analyze the changes in levels of metabolites in response to exogenous auxin, plants were hydroponically treated with IAA for 60 min. The individual plants at DAG 10 were transferred to a sterilized MS liquid medium without sucrose and vitamins from the plate culture after being washed with sterilized water, and held overnight in the same growth chamber. The next day, plants were transferred to a MS medium containing 10^{-8} M or 10^{-7} M IAA for 60 min. The control plants were treated with the corresponding concentrations of methanol. After 60 min IAA treatments, plants were immediately divided into shoots and roots, and both tissues were frozen with liquid nitrogen and stored at −80°C until extraction of metabolites.

Extraction and determination of metabolites

Metabolites were extracted following the methods of Sato et al. (2004) with slight modifications. Twenty to hundred milligrams of frozen shoots or roots were ground with a sample crusher (model SH-48; KURABO Industries, Ltd., Osaka, Japan) for 15 s four times with zirconia beads at 1,200 rpm. It is noted that each single sample is containing about more than 5 plants for shoots and 20 plants for roots. A methanol/chloroform/H_2O=1/1/0.4 solution was used for the extraction from all plant tissues. Ice-cold methanol (500 μl) containing 8 μM MeS and pure water (200 μl) containing 8 μM C10F were added and mixed using a vortex for 5 min at 4°C. Then, 500 μl chloroform were added and mixed using a vortex for 5 min at 4°C and the mixtures were centrifuged at 15,000×*g* for 5 min at 4°C. Five hundred microliters of supernatants were poured onto an ultrafiltration filter (Amicon Ultra 10,000MW, Millipore, Billerica, MA, USA) and centrifuged again at 9,000×*g* for 30 min at 4°C. The filtrate was dried out with a centrifugal concentrator CVE-100 (EYELA, Tokyo, Japan) connected with freeze dry system (LABCONCO Co. Model 77400, MO, USA). The residue was dissolved in 20 μl (roots) or 40 μl (shoots) of pure water.

The concentrated extracts from shoots or roots were analyzed using capirally electrophoresis-electrospray ionization mass spectrometry (CE; Agilent Technologies, Waldbronn, Germany, TOF-MS; Agilent Technologies, Palo Alto, CA, USA) equipped with a fused silica capillary column (100 cm×0.05 mm i.d., TSP050375, Polymicro Technologies, Phoenix, AZ, USA). The mass spectrometer was time-of-flight and set to scan *m*/*z* 50–1,000 in the full scan mode. The ion source temperature and voltage of MS for metabolite analysis was maintained at 300°C and 3,500–4,000 V, respectively. The concentrations of primary metabolites were calculated by the peak area of MeS for cationic analyses or C10F for anionic analyses. A total of 27 cationic compounds and 22 anionic compounds as primary metabolites were calculated. As the cationic metabolites, the concentration of 17 amino acid standard mixture ampule (Agilent Technologies, Waldbronn, Germany) containing Gly, Ala, Ser, Pro, Val, Thr, Ile, Leu, Asp, Lys, Glu, Met, His, Phe, Arg, Tyr and Cys were prepared at a concentration of 100 μM by diluting with pure water before use. Other cationic compounds were prepared at a concentration of 100 μg ml^{-1} each in 0.1 N HCl or pure water. All anionic metabolites were prepared at a concentration of 100 μg ml^{-1} each in pure water. All standard solutions were stored at −30°C until use.

Measurement of respiration rates

Respiration rates of shoots and roots were measured using liquid phase oxygen electrodes cuvette (DW2/2 Hansatech Ltd., King's Lynn, UK) at 25°C. Oxygen consumption rates were calculated assuming that the concentration in the air-saturated buffer was 253 μM at 25°C.

Reagents

MeS and C10F were purchased from WAKO (Wako Pure Chemical Industries Ltd. Osaka, Japan) and Tokyo Chemical (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan), respectively. Each stock solution was prepared in pure water or 0.1 N HCl, respectively. All chemicals were analytical or LC-MS grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA). Asp, adenine, adenosine, Gln, glutathione, Trp and uridine were purchased from Wako (Osaka, Japan). Cys, GABA and Orn were purchased from Sigma-Aldrich (St. Louis, MO, USA), Tokyo Chemical Industry Co. (Tokyo, Japan), Nacalai Tesque Inc. (Kyoto, Japan), respectively. Citrate, malate, 2OG, oxaloacetate, G6P, 6PG were purchased from Wako (Osaka, Japan). Pyruvate, *cis*-aconitate, *iso*-citrate, Ru5P, G1P, FBP, G3P, PEP, 2PG and 3PG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Succinate, fumarate, F6P, GAP and DHAP were purchased from Nacalai Tesque Inc. (Kyoto, Japan). R5P was purchased from MP Biomedical Inc. (Aurora, OH, USA).

Statistical analysis

Statistical analyses were performed using the Student's *t*-test ($p<0.05$) for paired comparisons, unless stated otherwise.

Results

Primary metabolites in the WT (Col-0) shoots and roots

Twenty amino acids were simultaneously determined

in the WT shoots and roots. The amount of amino acids ranged from 8.2±2.0 to 9,700±1,900 nmol g^{-1} fresh weight (FW) in the WT shoots (Figure 1A). Concentration of total amino acids in shoots was approximately 30 μmol g^{-1} FW. As in case of WT roots, Gln, Asn, Glu and Ser were also detected at high concentrations in WT shoots (Figure 1A). As the anionic compounds, organic acids and organic phosphates were measured at the same time. The concentration of malate was the highest (1,300±140 nmol g^{-1} FW). On the other hand, the *cis*-aconitate level was only 6.7±2.3 nmol g^{-1} FW. The levels of other organic acids detected ranged from 140±30 to 900±220 nmol g^{-1} FW in the WT shoots (Figure 1B). Oxaloacetate could not be detected possibly because it is degraded rapidly. The amounts of organic phosphates ranged from 30±8.3 to 510±90 nmol g^{-1} FW in the WT shoots (Figure 1C). When it was difficult to distinguish the phosphate compounds with the same molecular weight such as G6P, F6P and G1P, the peaks representing them were combined for quantification.

Amounts of amino acids ranged from 10±2.8 to 8,300±1,800 nmol g^{-1} FW in the WT roots (Figure 1D). Concentration of total amino acids in roots was approximately 30 μmol g^{-1} FW. WT roots contained high concentrations of Gln, Asn, Glu and Ser (Figure 1D). Among the anionic compounds, the concentrations of organic acids namely, malate, citrate and succinate in roots were much higher than those of other anionic metabolites (Figure 1E). The concentrations of malate, citrate and succinate were 4,200±890, 4,600±2,100 and 2,400±1,030 nmol g^{-1} FW, respectively. Levels of other organic acids detected ranged from 27.1±14 to 170±40 nmol g^{-1} FW in the WT roots. Oxaloacetate was not detected. Organic phosphates such as G6P, F6P and G1P were isolable detected. Their amounts ranged from 40±3.2 to 500±48 nmol g^{-1} FW in the WT roots (Figure 1F).

Distribution of primary metabolites between shoots and roots in WT plants

Certain organ-specific differences with regard to the levels of primary metabolites in WT plants were evident. Concentration of GABA in the roots was approximately 15-times higher than that in shoots. Levels of Thr, Val, Lys, Leu, Ile, His, Tyr and Trp in roots were 2- to 3-times higher than those in the shoots. Conversely, the Arg and Orn levels were found to be approximately 7- and 90-times higher in shoots than those in roots, respectively (Figure 1A, 1D).

Among the anionic compounds, the levels of malate and *cis*-aconitate were 3- to 4-times higher in roots than those in shoots, and those of succinate and citrate were 5- to 7-times higher in roots than those in the shoots. Whereas, the concentration of fumarate in shoots was found to be approximately 5-times higher than that in the roots. Organic phosphates in shoots and roots were contained at a comparable level (Figure 1C, 1F).

Changes in levels of primary metabolites in response to exogenous IAA in shoots and roots of WT

Changes in the levels of primary metabolites in *A. thaliana* WT due to a 60 min treatment with exogenous IAA (10^{-7}, 10^{-8} M) were monitored in 5 to 6 independent samples (Figure 1). Each sample contains *ca.* 20 plants in the roots and *ca.* 5 plants in shoots. Data revealed distinct metabolite as well as organ-specific patterns of changes in response to the two concentrations of IAA.

In the shoot tissue, the mean concentration of detected amino acids declined slightly under both IAA treatments except for Asp that slightly increased at 10^{-8} M IAA (Figure 1A). The levels of Gly significantly decreased due to both IAA treatments, whereas those of Ala significantly decreased at the lower IAA concentration. The other detected amino acids decreased in shoots at both the IAA concentrations; however, the changes were not statistically significant (Figure 1A).

The levels of organic acids and organic phosphates were altered in response to both the IAA treatments (Figure 1B, 1C). The levels of anionic metabolites except for succinate and *iso*-citrate declined at 10^{-8} M IAA in WT shoots. In contrast, those of pyruvate, fumarate and *iso*-citrate increased marginally at 10^{-7} M IAA. The levels of malate, citrate, 2OG and *cis*-aconitate showed a decrease at 10^{-7} M IAA in WT shoots, while those of succinate significantly increased at 10^{-7} M but not at 10^{-8} M IAA (Figure 1B). The levels of organic phosphates tended to decline at lower IAA concentrations; a decrease in the levels of G6P, F6P and G1P was observed at both IAA treatments (Figure 1C).

In the root tissue, GABA was the only cationic metabolite in the roots that significantly decreased due to 10^{-7} M IAA (Figure 1D). The mean concentrations of most cationic metabolites decreased in roots treated with both IAA concentrations except for Gly. The level of Gly increased at 10^{-7} M IAA (Figure 1D). Other cationic metabolites tended to decline due to both IAA concentrations without significance.

The levels of organic acids decreased at both IAA treatments in WT roots (Figure 1F). G6P levels significantly decreased due to both IAA concentrations. The other organic phosphates (F6P, G1P, G3P, R5P, Ru5P) showed a slight decline due to the treatment with both IAA concentrations; however, the differences were not significant (Figure 1F). The levels of 2PG, 3PG in shoots showed a tendency to increase at the higher IAA concentration with no significant difference (Figure 1C).

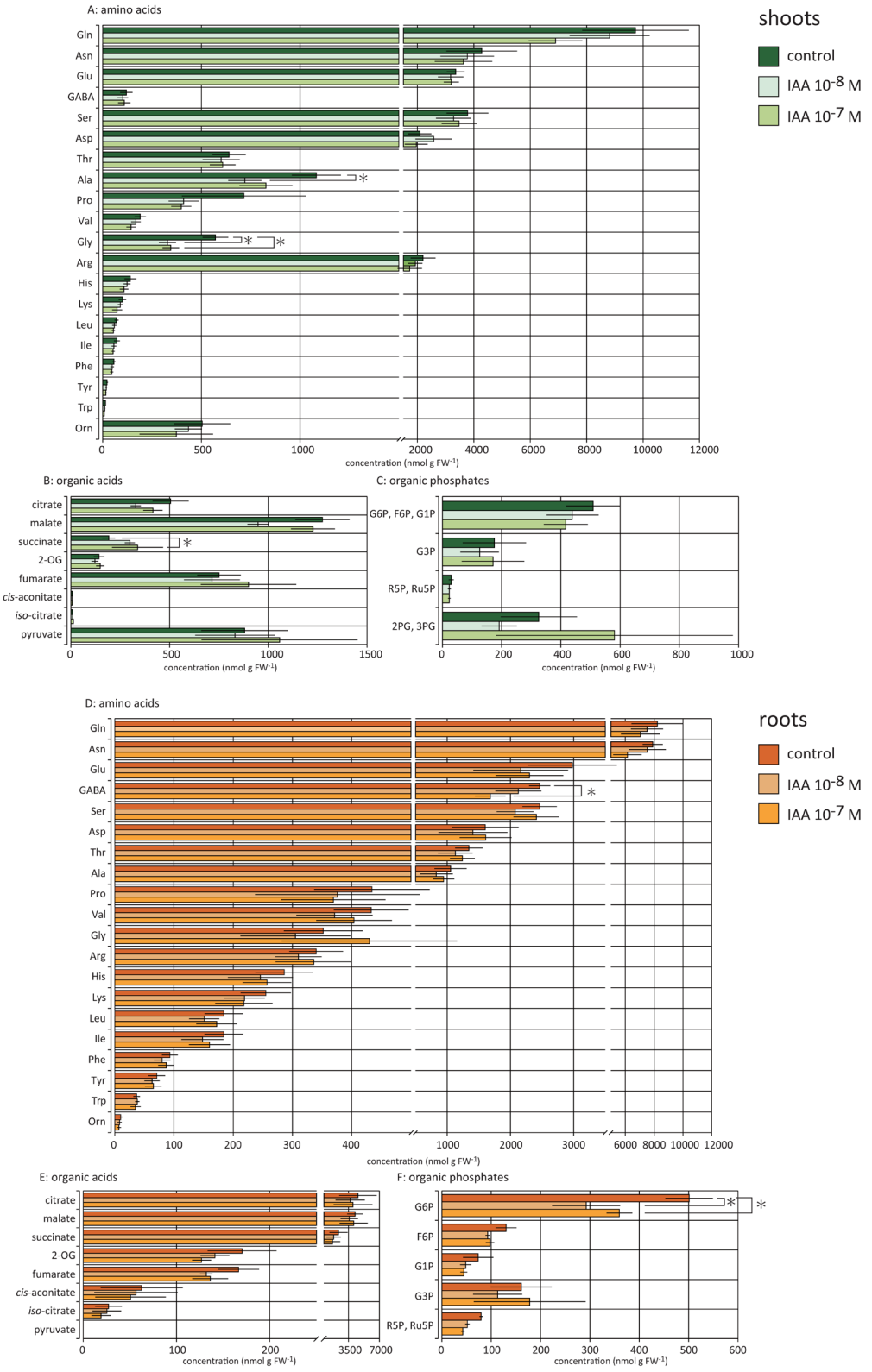


Figure 1. Changes in the levels of metabolites in response to exogenous IAA in WT plants. Changes in the levels of primary metabolites in response to exogenous IAA applied (10^{-7}, 10^{-8} M) for 60 min to 10-d-old WT plants of *A. thaliana*. A) amino acids in shoots, B) organic acids in shoots, C) organic phosphates in shoots, D) amino acids in roots, E) organic acids in roots, F) organic phosphates in roots. N.D. indicates not detected. Data represent mean±SE (n=5–6; Each sample contains *ca.* 20 plants in the roots and *ca.* 5 plants in shoots). Mark (*) denote statistically significant differences between control and IAA treatment plants detected by Student's t-test at $p<0.05$, respectively. The difference of x-axis scale in panel was deliberate in order to accommodate very small concentrations of some specific metabolites.

Primary metabolites in shoots and roots in auxin-signaling mutants, tir1*,* slr *and* arf7 arf19

In order to compare the effects of exogenous IAA on the levels of primary metabolites, we also measured the levels of primary metabolites in three auxin-signaling mutants, *tir1, slr* and *arf7 arf19* whose characteristic features have been described in the introduction.

The distribution pattern of metabolites in the mutants was not much different from that in the WT plants. The metabolite amounts in the mutants were found to be essentially comparable to those in the WT shoots and roots except for some metabolites in *slr*. Some amino acids such as Ala, Gln, Gly, Pro, Thr and organic acids showed high contents in *slr* plants compared to other plants. The high Pro content in *slr* plants (shoots and roots) might be the response to dehydration and osmotic stress (Yoshiba et al. 1997), since the *slr* mutation causes poor root system by blocking the lateral root and root hair formation (Fukaki et al. 2002).

The distribution pattern of amino acids in shoots of three mutants was found to be similar to that in WT plants except for a couple of exceptions (Figures 1 to 4). Thus, the level of Gly was detected double in *tir1* shoot compared to WT. Similarly, the concentration of Pro in *slr* shoots was 7-times higher than that in WT plants as was also the case in *slr* roots. Among the anionic metabolites in TCA cycle, the level of *cis*-aconitate in *tir1* shoots was found to be 2.4 times that of WT shoots (Figures 1B, 2B). The levels of malate, citrate and succinate in *slr* shoots were found to be approximately 2- to 4-times, whereas those of fumarate and *cis*-aconitate were found to be 11- and 14-times higher than those in WT shoots, respectively (Figures 1B, 3B, 6B). The concentrations of anionic metabolites, namely 2PG, 3PG and G3P in *tir1* shoots was found to be approximately 2 to 3 times lower than that in WT (Figures 1C, 2C, 6A). These compounds in *slr* shoots were found to be approximately 4 times lower than those in WT shoots (Figures 1C, 3C, 6A). The levels of 2PG, 3PG and G3P were detected 2 and 3 times lower in *arf7 arf19*, respectively (Figures 1C, 4C). The levels of total organic phosphates in auxin-signaling mutants seemed to be lower than those in the WT plants.

Changes in levels of primary metabolites in response to exogenous IAA in shoots and roots of three auxin-signaling mutants, tir1*,* slr *and* arf7 arf19

Subsequently, the changes in the levels of metabolites in response to IAA in auxin-signaling mutants namely, *tir1*, *slr* and *arf7 arf19* were measured. Interestingly, under IAA treatment, the variations of levels of most metabolites among the individual samples in case of the mutant plants were tend to be smaller than those in the WT plants (Figures 1 to 4).

The auxin-signaling mutants did not show any significant change in the mean concentrations of metabolites in shoots and roots due to the IAA treatments with a couple of exceptions. Thus, the level of 2PG, 3PG in *tir1* shoot significantly increased at 10^{-7} M IAA but not at 10^{-8} M IAA (Figure 2C). Also, the level of Gln in *slr* shoots significantly decreased due to the treatment with 10^{-7} M IAA concentration, but not 10^{-8} M IAA (Figure 3A). The mean concentrations of metabolites did not change in both of the shoots and roots of *arf7 arf19* due to the IAA treatments (Figure 4).

Effect of IAA on respiration in shoots and roots

The energy and substrates derived from respiration drive the primary metabolic pathway implying a strong relationship between the rate of respiration and levels of diverse metabolites. About half a century ago, IAA was shown to alter respiratory rates in the plant tissues depending on the concentration (Hackett and Thimann 1952; Mitchell et al. 1949). We measured the respiration rate in shoots and roots of WT and *arf7 arf19* after IAA (10^{-7} M) treatment for 60 min (Figure 5). The *arf7 arf19* was selected as a mutant which is deficient in the last step of the auxin-signaling pathway. Respiration rates in the shoots of WT and *arf7 arf19* were almost the same and did not change due to IAA treatment. However, the respiratory rate in WT roots significantly decreased at 10^{-7} M IAA but not in those of *arf7 arf19*.

Discussion

The levels of metabolites constitute the most important basis in determining the plant cellular metabolic status under the prevailing conditions. Metabolomics, a recently developed approach, offers an efficient means of comprehensively determining the levels of metabolites, also in response to the deviation from optimum growth conditions and diverse effector substances such as phytohormones. Such information obviously is of immense significance in understanding the regulation of plant growth and developmental processes. In the present study, we have addressed the question whether a short-term (60 min) exogenous IAA application would alter the levels of the metabolites in *Arabidopsis thaliana*. For the purpose, we compared the metabolites in WT and three auxin-signaling mutants (*tir1*, *slr* and *arf7 arf19*) employing CE-MS and characterized the responses thereof to exogenous IAA.

Distribution of primary metabolites in A. thaliana *shoots and roots*

Although some reports on metabolomics of *A. thaliana* are available, most of these are restricted to the analysis of shoots or whole seedlings. There is less information available about the roots (Hirner et al. 2006; Sulpice

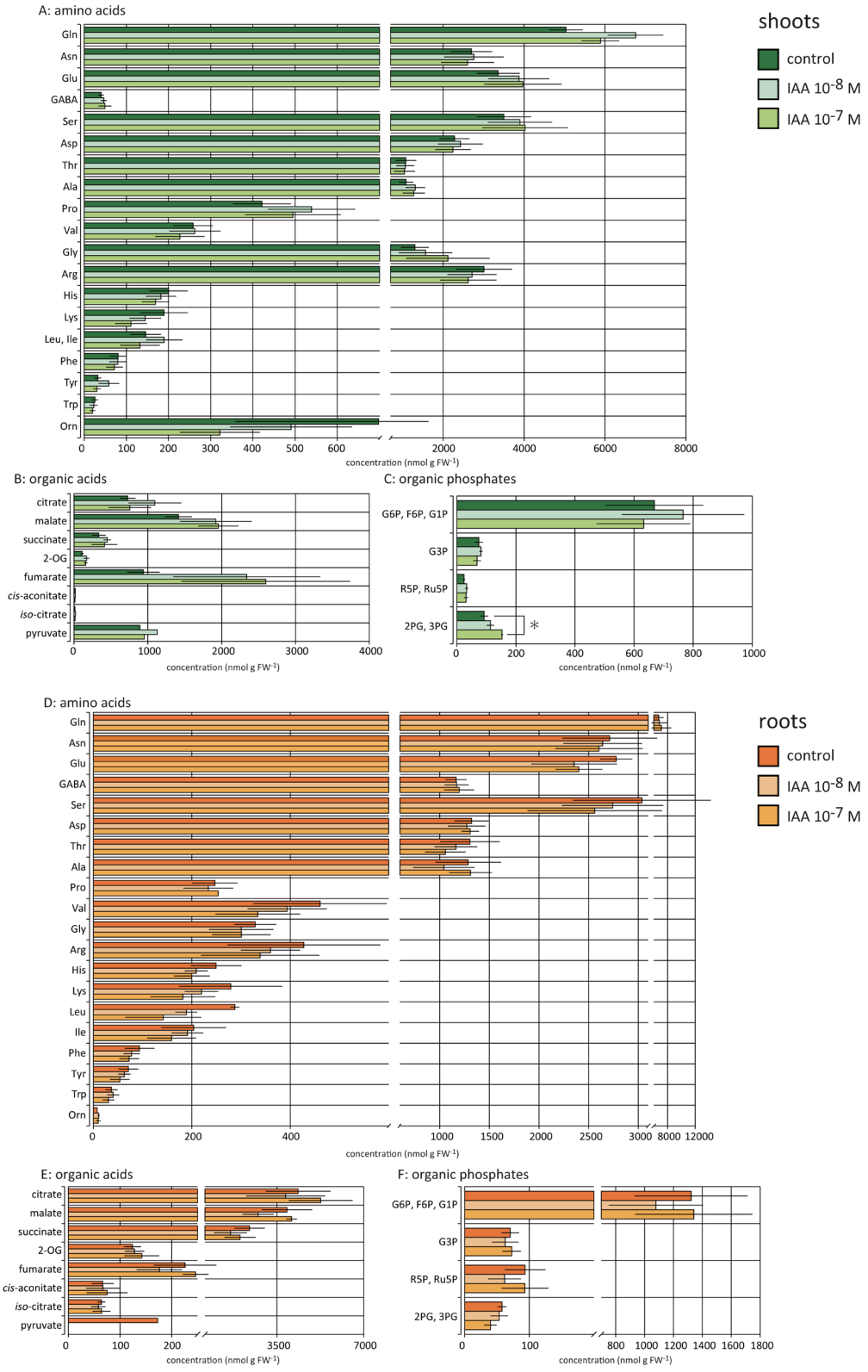


Figure 2. Changes in the levels of metabolites in response to exogenous IAA in *tir1* plants. Changes in the levels of primary metabolites in response to exogenous IAA (10^{-7}, 10^{-8} M) applied for 60 min to auxin-signaling mutant (*tir1*) plants of *A. thaliana*. A) amino acids in shoots, B) organic acids in shoots, C) organic phosphates in shoots, D) amino acids in roots, E) organic acids in roots, F) organic phosphates in roots. N.D. indicates not detected. Data represent mean±SE (n=3–4; Each sample contains *ca.* 20 plants in the roots and *ca.* 5 plants in shoots). Mark (*) denote statistically significant differences between control and IAA treatment plants detected by Student's t-test at $p<0.05$, respectively. The difference of x-axis scale in panel was deliberate in order to accommodate very small concentrations of some specific metabolites.

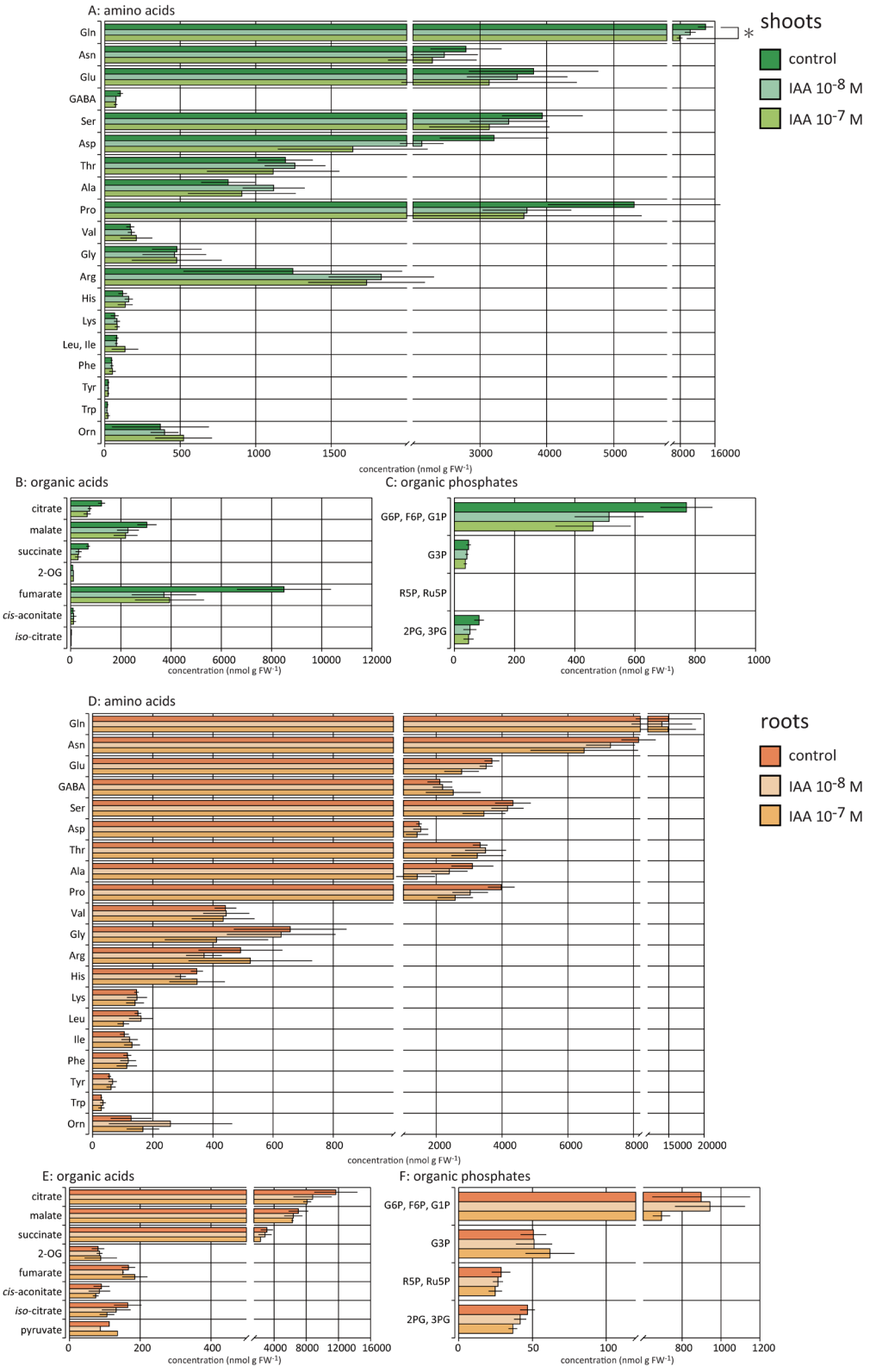


Figure 3. Changes in the levels of metabolites in response to exogenous IAA in *slr* plants. Changes in the levels of primary metabolites in response to exogenous IAA (10^{-7}, 10^{-8} M) applied for 60 min to auxin-signaling mutant (*slr*) plants of *A. thaliana*. A) amino acids in shoots, B) organic acids in shoots, C) organic phosphates in shoots, D) amino acids in roots, E) organic acids in roots, F) organic phosphates in roots. N.D. indicates not detected. Data represent mean±SE (n=3–4; Each sample contains *ca.* 20 plants in the roots and *ca.* 5 plants in shoots). Mark (*) denote statistically significant differences between control and IAA treatment plants detected by Student's t-test at $p<0.05$, respectively. The difference of x-axis scale in panel was deliberate in order to accommodate very small concentrations of some specific metabolites.

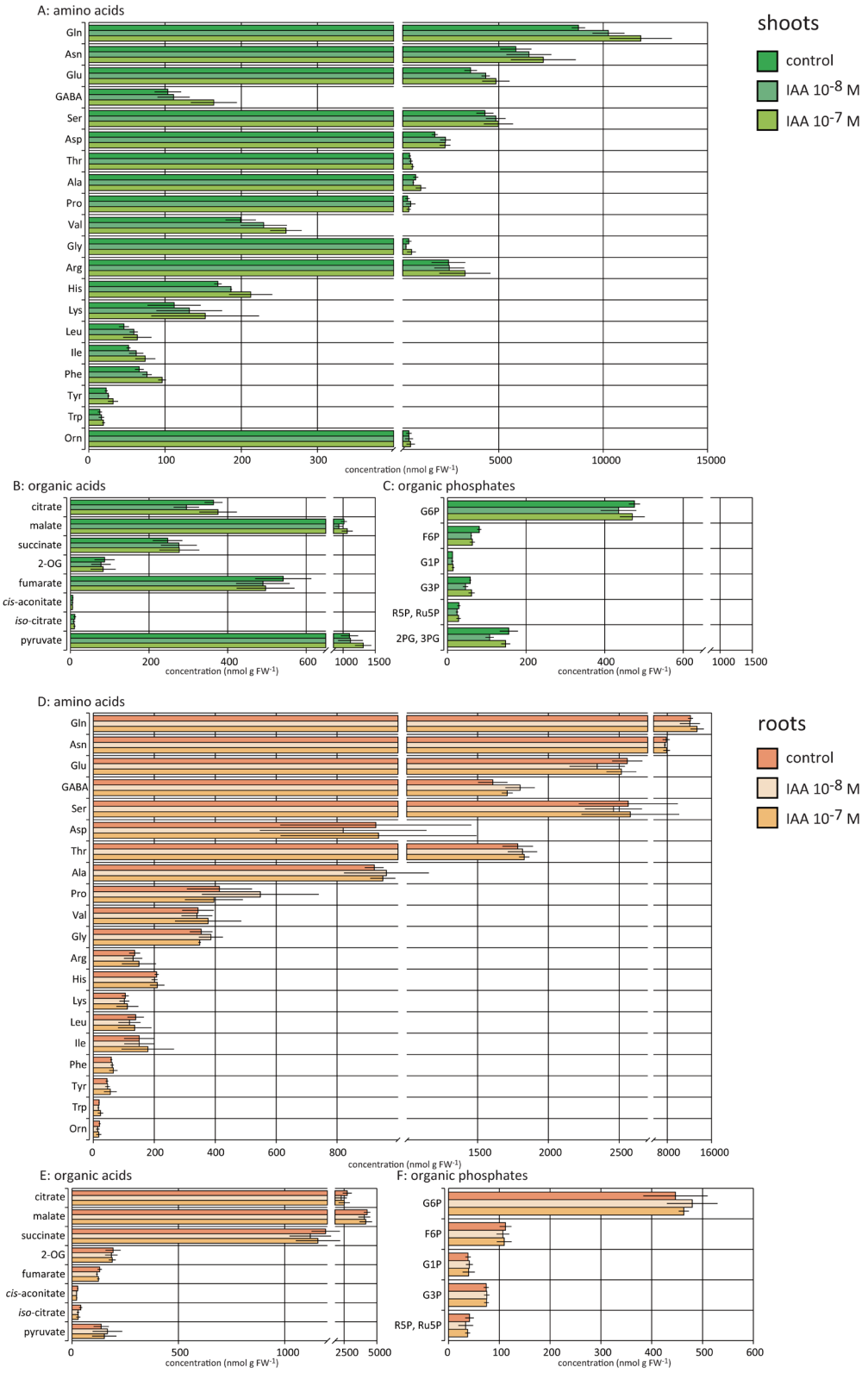


Figure 4. Changes in the levels of metabolites in response to exogenous IAA in *arf7 arf19* plants. Changes in the levels of primary metabolites in response to exogenous IAA (10^{-7}, 10^{-8} M) applied for 60 min to auxin-resistant double mutant (*arf7 arf19*) plants of *A. thaliana*. A) amino acids in shoots, B) organic acids in shoots, C) organic phosphates in shoots, D) amino acids in roots, E) organic acids in roots, F) organic phosphates in roots. N.D. indicates not detected. Data represent mean±SE ($n=3$; Each sample contains *ca.* 20 plants in the roots and *ca.* 5 plants in shoots). Mark (*) denote statistically significant differences between control and IAA treatment plants detected by Student's *t*-test at $p<0.05$, respectively. The difference of *x*-axis scale in panel was deliberate in order to accommodate very small concentrations of some specific metabolites.

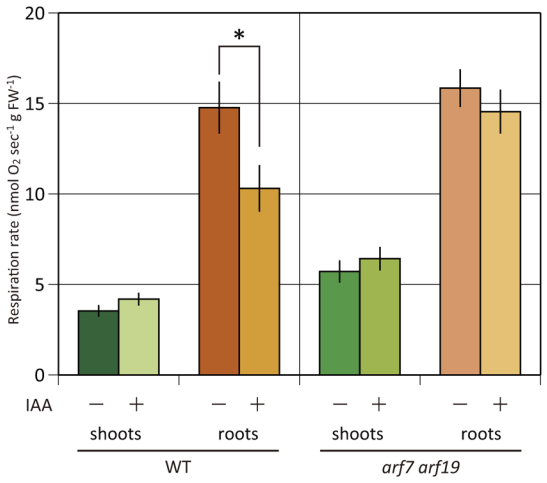


Figure 5. Effect of IAA on the rate of respiration. Effect of IAA (10^{-7} M) on the rate of respiration in shoots and roots of WT and *arf7 arf19* mutant of *A. thaliana*. Data represent mean±SE (n=6–8). Mark (*) denotes statistically significant differences between control and IAA treated plants detected by Student's t-test at $p<0.05$.

et al. 2010; Tschoep et al. 2009). Furthermore, the measurements of metabolites of *Arabidopsis* roots by CE-MS, a technique particularly suitable for the analysis of polar metabolites, are quite limited (Watanabe et al. 2010).

To begin with, we compared the contents and distribution of metabolites between shoots and roots of WT and auxin-signaling mutant plants grown in the hydroponic culture. The levels and distribution of amino acids were almost the same in WT and mutant plants except for some metabolites in *slr* (Figures 1 to 4). The concentration of Glu accounted for 20 to 30% of total amino acids in shoots and roots that conforms to previous studies (Armengaud et al. 2009; Hirner et al. 2006; Watanabe et al. 2010). Both the shoots and roots contained high levels of Gln, Glu, Asn and Ser in WT and the mutants. The total amino acid contents (20 to 50 μmol g^{-1} FW) observed in the present study are similar to those reported previously (20 to 100 μmol g^{-1} FW) (Armengaud et al. 2009; Hirner et al. 2006; Rawat et al. 1999; Watanabe et al. 2010). The concentrations of Gln in shoots and roots (9,700 and 8,200 nmol g^{-1} FW, respectively) and Glu in shoots and roots (3,400 and 3,000 nmol g^{-1} FW, respectively) in the present study are 10- to 30-times higher as compared to those reported in previous studies on *A. thaliana* (Hirner et al. 2006; Watanabe et al. 2010). However, our results are consistent with those of Armengaud et al. (2009) and Rawat et al. (1999) involving the analysis of hydroponically grown plants. Apparently, the higher contents of Gln and Glu might be ascribed to some factor(s) associated with culture and growth conditions of plants.

Concerning the anionic compounds, most previous studies have presented only the relative values of anionic metabolites to WT or control rather than the absolute amounts (Cook et al. 2004; Nikiforova et al. 2005; Sudre et al. 2013). Likewise, the levels of organic acids and organic phosphates in *Arabidopsis* have been mostly reported on whole plant basis (Armengaud et al. 2009; Scheible et al. 2004). There are only a few studies providing data on organ-specific break-up of the levels of anionic metabolites such as organic acids and organic phosphates. This appears partly to be due to the limitations of methods employed such as HPLC or GC-MS in the measurement of metabolites. Watanabe et al. (2010) determined the ratios of primary metabolite levels including organic acids and organic phosphates in WT and a mutant (alternative oxidase knockout transgenic line, *aox1a*) of *A. thaliana* under low-N stress using CE-MS. Our data differed a little from those of Watanabe et al. (2010) concerning some metabolites. For example, the root malate and citrate levels were 1.5-times greater while those of succinate were 3-times greater in their study compared to ours. The levels of other organic acids were comparable. In case of shoots, the levels of organic acids namely, fumarate, malate and *cis*-aconitate were lower in our study than those reported by Watanabe et al. (2010). It may be due to a difference in the methods of analysis and plant growth. We, however, are not able to assign specific reason(s) for the differences employing the same measurement technique in the two studies.

IAA-induced changes in the levels of primary metabolites

Metabolomic analysis of plants is exceedingly gaining popularity in researches addressing diverse issues of concern (Obata and Fernie 2012). However, there are fewer reports concerning the effects of plant hormones on the dynamics of metabolites. It is quite probable that the levels of primary metabolites did not change under hormone treatments to the detectable extent owing largely to the existence of a strong cellular homeostatic control of primary metabolism. On the other hand, in the present study, we noticed that the intra-sample deviations of the levels of metabolites due to IAA-treatment of WT plants were much larger than those in the similarly treated auxin-signaling mutants (Figures 1 to 4). Such difference suggests a certain IAA-dependent effect on metabolism.

Possible regulation of metabolic pathways by IAA

The observed changes in measured primary metabolites are represented in a metabolic pathway map (Figure 6).

The levels of G6P in the glycolysis decreased in the roots of IAA treated WT plants but not in those of mutant roots. Some organic phosphates such as DHAP, GAP, FBP and PEP in glycolysis were below the detection limits in our experiments both in WT and auxin-signaling mutant plants. Organic phosphate in mutant plants seemed to be found easier than WT

plants, whereas pyruvate could not be detected in WT roots and *slr* shoots (Figures 1E, 3B, 6A). Pyruvate is one of key metabolic intermediates. This is the end product of glycolysis and the starting metabolite to flow TCA cycle, amino acids metabolism and biosynthesis of other metabolites such as lipids or terpenoids. Pyruvate levels fairly fluctuated between tissues or mutant lines. The auxin signaling may strongly affect on the metabolic flow to incoming and outgoing of pyruvate as a key intermediate in plant primary metabolism. Instead, the levels of other metabolites seem to be maintained under robustness. This is the future subject. Decrease of sugar phosphates including G6P in the glycolysis by IAA treatments in WT roots suggested that their metabolism might be regulated by auxin-responsive genes, since these changes in response to IAA were not observed in the auxin-signaling mutant roots.

Among the anionic metabolites in the TCA cycle, the levels of succinate showed difference response between shoots and roots in WT plants (Figure 6B). Succinate and succinate dehydrogenase (SDH) is known to be a TCA marker reported in Lendvai et al. (2014). In our study, the decline of succinate level is well-correlated to the changes in the respiration in response to exogenous IAA in WT roots, but not in WT shoots (Figures 5, 6). Other organic acids namely, citrate, *cis*-aconitate, *iso*-citrate, 2OG and fumarate showed clear tendencies to decrease under IAA treatments in WT roots. Some amino acids such as Lys, Alg, Gln and organic acids such as malate and citrate are known to be accumulated in the vacuole (Etienne et al. 2013; Tohge et al. 2011). The distribution of these metabolites in the cell is under analysis.

The levels of GABA showed statistically significant differences between the roots of control and IAA (10^{-7} M) treated WT plants. On the other hand, the levels of GABA did not change due to IAA treatments in WT shoots (Figure 6A). Even in the auxin-signaling mutants, the level of GABA slightly altered in response to exogenous IAA. This might be related to the polyamine synthesis as reported in previous studies (Kishinami 1988; Park and Lee 1994; Tiburcio et al. 1997).

In WT shoots, some amino acids such as Pro, Leu, Ile, Asn, Tyr, Trp, Phe, Val and Gln decreased due to treatment with both IAA concentrations except for Gly and Ala (Figure 1A, 6A). However, the changes in these amino acids in response to IAA did not show significant differences compared to control plants.

IAA-dependent changes in the rate of respiration

Although there are few studies concerning the effect of IAA on metabolism in plants, it was reported about half a century ago that the IAA treatment affected the increase/decrease in the respiration of tomato stem and *Avena* coleoptile (Commoner and Thimann 1941; Hackett and Thimann 1952; Mitchell et al. 1949). In our experiments, the respiration rates in WT roots under IAA treatment significantly decreased compared to those in control while remaining unchanged in the shoot (Figure 5). It is thus quite likely that the levels of decreased amino acids such as Glu, Gln and Asn due to IAA treatments are a direct consequence of the suppressed respiration in WT roots.

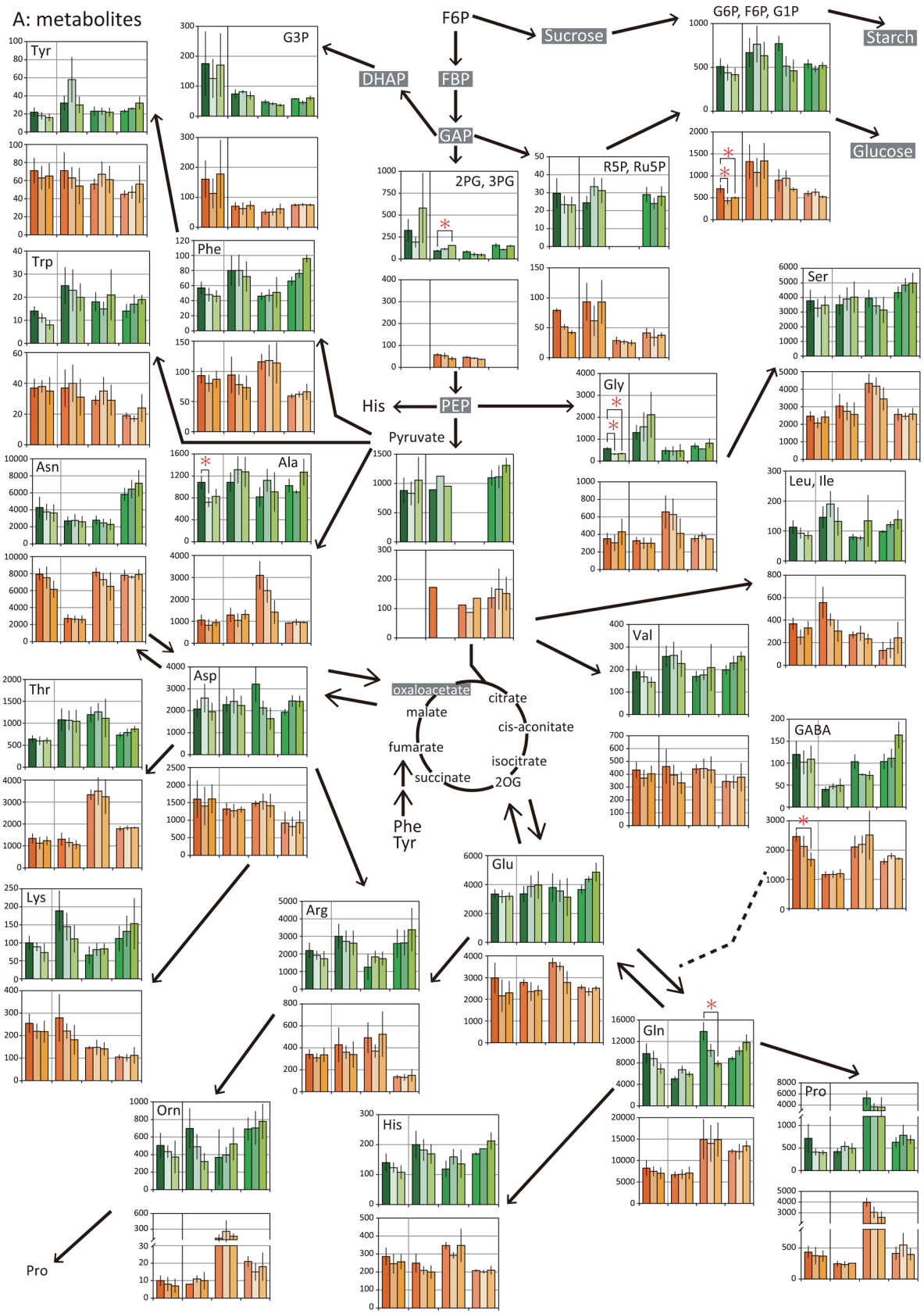
In contrast to the WT plants, the respiration rate in both *arf7 arf19* shoots and roots did not change under IAA treatment. This might account to some extent for the unaltered levels of above metabolites in *arf7 arf19* in response to exogenous IAA.

Gene expression and changes in levels of metabolites

As the initial target events of IAA, gene expressions have been extensively studied. Armstrong et al. (2004) showed that 10% of the auxin-responsive genes were involved in metabolism of *Arabidopsis* in treated with 5 μM IAA for 1 h. Similar changes in genes related to cellular metabolism have been reported in plants treated with IAA (Goda et al. 2004; Okushima et al. 2005; Singla et al. 2007). The IAA-induced changes in the levels of metabolites could be ascribed to altered changes in expression of appropriate genes induced by the auxin treatment.

We surveyed the link between the changes in primary metabolites and the expression of genes regulated by auxin, considering the available comprehensive microarray data from previous studies. Laskowski et al. (2006) showed that the expression of genes encoding aspartate aminotransferase (AST), phosphate metabolism and NADP dependent malic enzyme were induced by treatment with 10 μM IAA (45 min to 12 h) in roots of *A. thaliana*.

In our experiments, several metabolites related to AST underwent changes of varying magnitude. Thus, Glu levels decreased due to both IAA treatments in WT roots, while those of Asp decreased in WT roots at 10^{-8} M but not at 10^{-7} M IAA (Figure 6A). A decrease in 2OG was evident at both IAA concentrations in WT roots. In WT shoots, the level of Gln showed a tendency to decline at both IAA treatments (Figure 6B). The levels of Asp increased at treatment with 10^{-8} M IAA and did not change at 10^{-7} M IAA in WT shoots (Figure 6A). The observed changes in primary metabolites in response to IAA might be caused by induced gene expression of AST. Since these changes were also evident in auxin-signaling mutant plants, auxin-dependent regulation might rely on different auxin signaling pathways. There are some reports available concerning the auxin dependent stimulation of the expression of genes related to phosphate metabolism (Goda et al. 2004; Laskowski et al. 2006). In the present study, the concentrations of G6P significantly decreased in WT roots, but not in those of



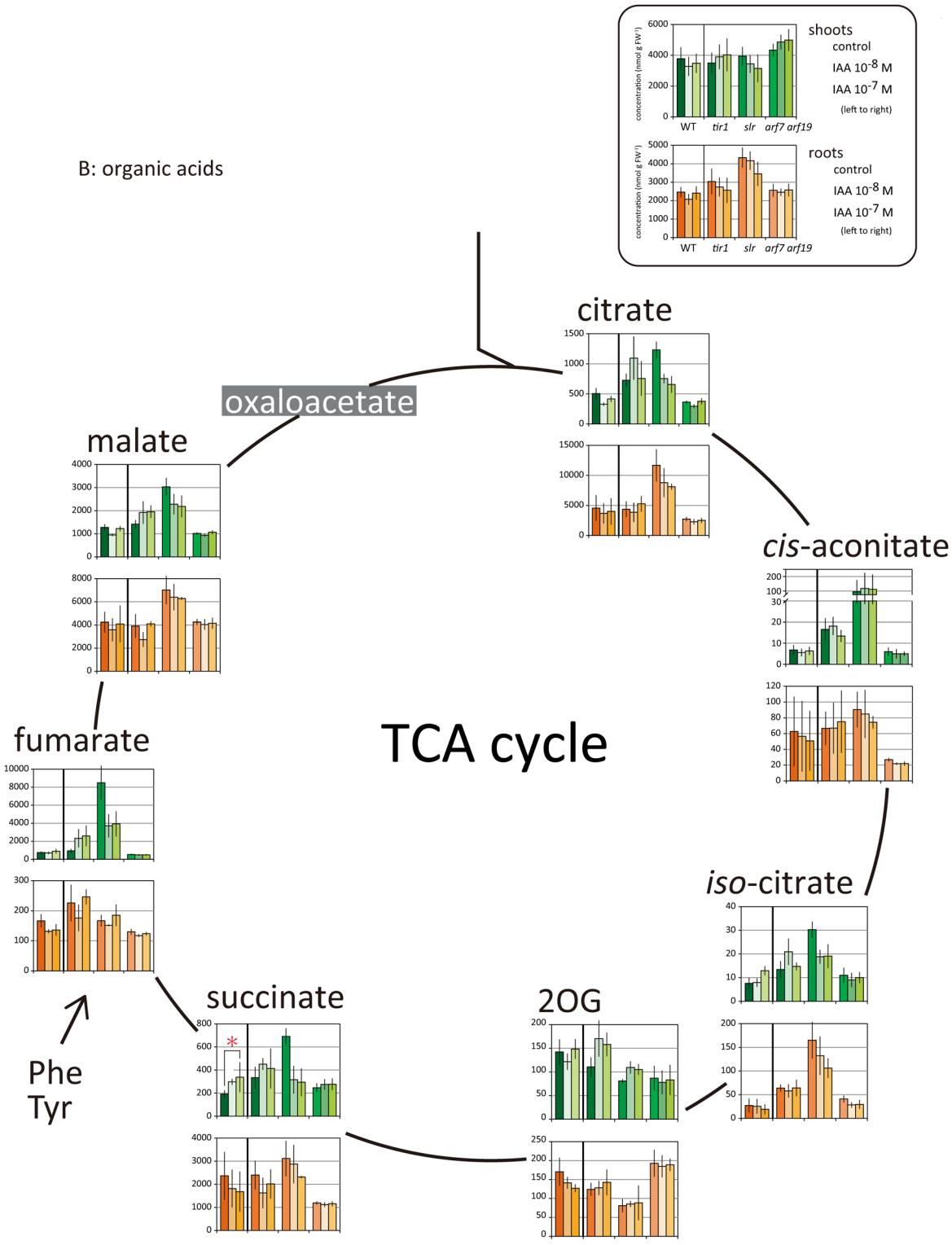


Figure 6. Changes in the levels of metabolites described in metabolic pathway. Metabolic pathway map representing the altered primary metabolites in response to IAA treatments for 60 min in *A. thaliana* WT (n=5–6), *tir1* (n=3–4), *slr* (n=3–4) and *arf7 arf19* (n=3). The changes of metabolites in response to IAA in shoots and roots of plants were shown upper column and lower column, respectively. The bar details are described in right upper side on Figure 6B. White column and white text indicate 'not detected' or 'under detection limits.' Mark (*) denotes statistically significant differences between control and IAA treated plants detected by Student's t-test at $p<0.05$.

the auxin-signaling mutants (Figure 6A). Consequently, the involvement of those genes in the regulation of organic phosphate metabolism might be suggested.

The altered expression of genes involved in metabolism in response to auxin seems to have affected a change in the levels of metabolites in the WT plants. The expression of those genes was repressed in *arf7 arf19* mutant even following the treatment with IAA (Okushima et al. 2005). This is consistent with our findings of most metabolites not changing in three auxin-signaling mutants in response to the exogenous IAA. Additionally, the less changes in the levels of metabolites in response to exogenous IAA observed in mutants also suggest the existence of regulation mechanisms related to auxin signal pathway (Figure 6). Relationship between hormonal regulation and robustness of metabolic pathway at the metabolite levels is an important future subject.

Author contributions

A.A. and T.M. designed research, performed research, analyzed data, and wrote the paper. M.O., K.I., C.S. and H.F. performed research and analyzed data. D.T. and C.M. contributed analytic tools and performed a part of research.

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