Enhancement of starch accumulation in plants by exogenously applied methyl jasmonate

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Title

Enhancement of starch accumulation in plants by exogenously applied methyl jasmonate

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Short title

Starch accumulation by methyl jasmonate

Abstract

Increasing starch production is a central issue in plant biology and applied biotechnology. Although genetic engineering has been applied to produce plants containing much starch, chemicals that promote starch accumulation have not been well studied. Here, we report that exogenously applied methyl jasmonate (MeJA) enhanced the leaf starch content of *Arabidopsis thaliana*. A significant increase in starch production was detected during the light period after *Arabidopsis* was treated with high doses of MeJA (100 - 1000 μM). The MeJA application influenced starch production rather than starch degradation because the expression of starch biosynthetic genes was upregulated by MeJA. The promotion of starch accumulation by MeJA was demonstrated not only in *Arabidopsis* but also in tobacco and spinach. These results suggest that the promotion of starch accumulation by MeJA is a common response found in a variety of plants.

Key words

Arabidopsis thaliana, methyl jasmonate, spinach, starch, tobacco

Introduction

Starch is a major storage carbohydrate in plants. Since starch is used for a wide variety of applications including food, feed, fuel, and industry, technical developments that increase the starch yield of plants are considered very important (Slattery et al. 2000; Smith 2008). Starch accumulates in both photosynthetic and nonphotosynthetic tissues. The chloroplasts of leaves contain transitory starch, which is synthesized in the day and is broken down at night. The nonphotosynthetic storage organs such as tubers, roots, and seeds have reserve starch. Because the amount of reserve starch in

the storage organs is overwhelming, almost all starch applied to end-uses is the reserve type (Slattery et al. 2000; Smith 2008; Keeling and Myers 2010). However, leaf biomass containing much transitory starch can be a promising source of biofuel if biorefinery technologies can be developed (Smith 2008).

The precursor of starch synthesis is ADP-glucose, which is converted from glucose 1-phosphate by ADP-glucose pyrophosphorylase (AGPase) (Zeeman et al. 2010; Geigenberger 2011; Stitt and Zeeman 2012). ADP-glucose is used as a substrate of starch synthase (SS), which generates linear α-1,4 glucosyl chains. Starch branching enzyme (SBE) and debranching enzyme (DBE) are involved in the formation of starch granules. Light-dependent redox signals, metabolic intermediates, and phosphate concentration influence starch biosynthesis (Geigenberger et al. 2005).

Biotechnological approaches to enhancing the reserve starch accumulation have been attempted for two decades (Slattery et al. 2000; Smith 2008; Keeling and Myers 2010). Generally, potato (*Solanum tuberosum*) tubers have been used for this purpose. The first success was obtained by overexpressing the AGPase gene from *Escherichia coli* in the potato (Stark et al. 1992). The transformants had an average of 35% more tuber starch than the control plants. However, this effect is not likely universal, because such magnitudes of increase have not been recorded in other potato varieties, cassava, or maize (Sweetlove et al. 1996; Smith 2008). The most effective strategy to increase the starch accumulation of the potato was the elevation of the ADP-glucose contents in the tuber plastid (Geigenberger 2011). Overexpression of the adenylate transporter gene and downregulation of the adenylate kinase gene could increase the ADP-glucose levels in the tuber. As a result, the tuber starch contents of transgenic potatoes increased up to two-fold compared to those of control potatoes. Similar effects have been demonstrated in field trials.

Enhancement of the transitory starch contents has been achieved by blocking the starch breakdown pathway. Genes involved in the degradation of transitory starch have been identified using the molecular genetics of *Arabidopsis thaliana*. A starch-excess phenotype was observed when

the glucan-water dikinase (GWD) gene was deficient (Lloyd et al. 2005). Downregulation of GWD genes enhanced starch accumulation in fodder crops such as clover (*Trifolium repens*), alfalfa (*Medicago sativa*), ryegrass (*Lolium perenne*), and silage maize (*Zea mays*) (Zeeman et al. 2010).

Despite increasing reports on the enhancement of the starch yield via genetic manipulation, chemicals that promote starch accumulation have not been developed. Such reagents may become convenient tools to simply increase the starch content in plants to which breeding and genetic manipulation are not easily applied. In this paper, we report that methyl jasmonate (MeJA), which is a plant growth regulator, enhanced the leaf starch contents in the aerial parts of plants by upregulating the expression of starch biosynthetic genes.

Materials and methods

Plant materials and methyl jasmonate (MeJA) treatment

A. thaliana (L.) Heynh (ecotype Columbia), tobacco (*Nicotiana tabacum* L. cv. Samsun), and spinach (*Spinacia oleracea* L. cv. Solomon; Sakata Seed, Yokohama, Japan) were grown in 7-cm plastic pots filled with Peatban (Sakata Seed). Plants were grown in the growth chamber (NK System, Tokyo, Japan) with 100 μmol m^{-2} s^{-1} light under a long-day condition (16 h light/8 h dark cycle) at 22°C. The density of planting was three plants per pot. MeJA (Wako, Osaka, Japan) was dissolved in ethanol at the concentrations of 0 (ethanol only), 100, 250, 500, and 1000 mM, respectively. Ten micro liters of the corresponding MeJA solutions were added to water (9.99 mL). The resulted solutions (0, 100, 250, 500, and 1000 μM MeJA) were used for the MeJA application. The MeJA solutions were sprayed on the surface of the leaves of three-weeks-old plants with a hand-pump aerosol spray bottle (1 mL per pot) at the start of the light period. The aerial parts of the plants were harvested at the end of the light period in the same day and stored at -70°C until use.

Quantification of starch content

The starch content was measured as described previously (Takahashi et al. 2012). Frozen tissues were treated twice with 10 volumes of 80% (v/v) ethanol at 80°C for 20 min. The ethanol insoluble residue was extracted by an equal volume of 0.4 M KOH at 80°C for 60 min. After the extract was neutralized, soluble starch was digested by 10 U α-amylase and 7 U amyloglucosidase. Glucose formation was determined by a glucose oxidase- and peroxidase-based enzyme assay. Starch content was calculated based on the released glucose. Recoveries determined by the standard addition method were applied to calculate the content of starch.

For whole plant starch staining, the areal parts of the plants, which were decolorized in hot 80% (v/v) ethanol, were stained with a solution containing 10 mM I_2 and 14 mM KI for 10 min at room temperature. After the plant was rinsed with water, photographs were taken.

Anthocyanin analysis

Leaves were ground to powder in liquid nitrogen. The sample was transferred to 300 μL of 1% (v/v) HCl in methanol and extracted at 4°C for 12 h. Deionized water (200 μL) and chloroform (500 μL) were added and mixed. The mixture was centrifuged at 15,000 *g* for 5 min at 4°C. The top layer (400 μL) was transferred into a new tube and 600 μL of 1% (v/v) HCl was added. After centrifugation at 15,000 *g* for 5 min at 4°C, the absorbance of the supernatant was measured at 530 nm (A_{530}) and 657 nm (A_{657}) by a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). The absorbance of anthocyanin was calculated as A_{530} -A_{657} (Martin et al. 2002).

Gene expression analysis

The transcript levels of starch metabolism genes were analyzed by a reverse transcription-polymerase chain reaction (RT-PCR) system (AMV Reverse Transcriptase XL; Takara Bio, Shiga, Japan). Total RNA was extracted from the aerial parts of the plants with the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). Five hundred nanograms of total RNA were used. Reverse transcription was performed at 45°C for 30 min. The PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s per cycle. The primers and cycles of PCR were denoted in Supplementary Table 1. The amplified products were analyzed by 1% agarose gel electrophoresis, and the results were documented by a LAS-4000 Image Analyzer (Fujifilm, Tokyo, Japan). After the band intensity was determined using ImageJ software (http://rsbweb.nih.gov/ij/), the relative amounts of the transcripts were calculated by standardizing the band intensities at zero time.

Statistical analysis

Data for *P* values were analyzed by Student's *t* test at a significance level of 0.05.

Results and discussion

Our first investigation was to find candidate chemicals that promote starch biosynthesis. We searched databases such as the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and the AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp) for the gene expression of *Arabidopsis* and found that MeJA enhanced the transcript accumulations of *SS* genes, such as *granule bound SS 1* (*GBSS1*, At1g32900), *SS 2* (*SS2*, At3g01180), and *SS 3* (*SS3*, At1g11720). This suggests that MeJA affects starch synthesis in *Arabidopsis*.

In order to test whether MeJA changes starch contents in *Arabidopsis* leaves, we sprayed MeJA

solutions on the areal parts of the 3-week-old plants. In the preliminary experiment we applied 1 and 10 μM MeJA solutions, because these concentrations were used in the experiments involving the corresponding databases (Arabidopsis eFP Browser; 10 μM, AtGenExpress Visualization Tool; 1 μM). However, we could not find significant alterations of the starch contents due to the MeJA additions at both concentrations (data not shown). We thereafter increased the MeJA doses up to 1000 μM, which is an extraordinary level. Figures 1a and b show the starch accumulation in the areal parts of plants to which MeJA was added at concentrations ranging from 100 to 1000 μM. The following tests were done using plants harvested at the end of the light period. Starch staining of MeJA-treated plants was apparently stronger than that of control plants (Fig. 1a). The measurement of starch contents in the areal parts of plants indicated that the administration of MeJA significantly increased the starch accumulation at all concentrations tested (Fig. 1b). The leaf starch content of the 1000 μM MeJA-treated plant reached a level approximately two-fold higher than that of the control plant. The plants treated with 500 μM MeJA showed a greater elevation of starch contents in the light period than the control plants (Fig. 1c). These results indicate that the high doses of MeJA promoted starch accumulation as a result of the enhancement of starch synthesis. Jasmonic acid (JA) also increased starch content although the effect was somewhat weaker at lower concentration (Supplementary Fig. 1).

Although the enhancing effect of MeJA on the starch content was significant at the end of the first light period after the MeJA addition, the effect was attenuated at the ends of the second and later light periods (Fig. 2a). Conversely, anthocyanin accumulation, which is a typical response to MeJA in plants (Franceschi and Grimes 1991; Shan et al. 2009), started two days after the treatment, i.e. at the end of the third light period (Fig. 2b). The addition of 500 μM MeJA little influenced the growth of the areal part of *Arabidopsis* (Fig. 2c).

We measured the transcript levels of starch metabolism genes such as *AGPase* isoform genes (*APS1*, *APL1*, and *APL4*), *SS* genes (*GBSS1*, *SS2*, and *SS3*), an *SBE* gene (*SBE3*), *isoamylase* gene

(*ISA1*), *GWD* gene (*GWD1*), and *β-amylase* gene (*BAM3*) in the 500 μM MeJA-treated and control *Arabidopsis* plants (Fig. 3). For a positive control, the *VSP1* gene which has been characterized as the jasmonate-responsive gene (Guerineau et al. 2003) was used. The actin gene (*ACT2*) was analyzed as a constitutively expressed gene. In Fig. 3, statistical judgment was not performed, because the data were obtained by the semi-quantitative method. There was a tendency for the expressions of starch synthetic genes, i.e. *APS1*, *APL4*, *GBSS1*, *SS2*, and *SS3*, to be upregulated by the MeJA administration. This suggests that MeJA enhanced starch accumulation in *Arabidopsis* by regulating the expressions of several starch biosynthetic genes. Investigation of the SS2 promoter by using the PLACE Web site (http://www.dna.affrc.go.jp/PLACE/) indicates that the promoter contains one MYCATRD22 site (CACATG, -43 bp in antisense orientation) and two T/GBOXATPIN2 sites (AAACGTG, -247 bp and -354 bp in sense orientations), respectively. The MYCATRD22 and T/GBOXATPIN2 sites are jasmonate responsive elements which have been characterized previously (Lorenzo et al.2004; Boter et al. 2004). The *APS1* gene also has one MYCATRD22 site (-413 bp in antisense orientation) in its promoter region. These genes might be up-regulated via the known jasmonate signaling pathways.

Finally, we investigated whether the promoting effect of MeJA on starch accumulation is exhibited not only in *Arabidopsis* but also in other plant species. Tobacco and spinach were treated with MeJA (100 and 500 μM), and then their leaf starch contents were measured. Figure 4 shows that MeJA significantly enhanced starch accumulations in both species. Similar results were obtained when MeJA was administered to alfalfa (*Medicago sativa*), cucumber (*Cucurbita sativa*), and wheat (*Triticum aestivum*) (data not shown). This shows that MeJA increases the starch contents in many plant species.

The promotion of starch accumulation has been achieved by controlling the expression of genes related to starch metabolism (Slattery et al. 2000; Smith 2008; Keeling et al. 2010). The development of chemicals which promote starch accumulation is also important in practical use, because such

compounds are convenient for enhancing the starch production of plants which have difficulty in breeding and genetic manipulation. A recent report noted that volatile chemicals emitted by microbes enhanced leaf starch accumulation, although the active compounds were not identified (Ezquer et al. 2010). Exogenous asparagine enhanced starch accumulation in lupin seeds (Borek et al. 2013). These reports hypothesize that the exogenous application of chemicals may enhance starch synthesis in plants. Here, we found that MeJA is an inducer of starch accumulation. MeJA shows many physiological responses related to developmental processes and defense responses in a wide variety of plant species (Creelman and Mullet 1997; Wasternack 2007). However, there is no report that describes the enhancing effect of MeJA on leaf starch accumulation, as far as we know. It should be noted that the effective concentrations of MeJA were extremely high (100 - 1000 μM). This response to MeJA may be an artifact that does not occur under natural conditions.

MeJA enhanced the increase of leaf starch content during the light period (Fig. 1c). The maximum effect was observed within the first day of the MeJA administration (Fig. 2a). This suggests that one can treat MeJA to plants in the morning then harvest them before sunset of the day in the practical applications. MeJA elevated the expression of starch biosynthetic genes, but little influenced the expression of genes for starch degradation (Fig. 3). These findings show that MeJA promoted starch synthesis rather than inhibited starch breakdown. To increase the starch contents of forage and silage plants by genetic engineering, downregulation of starch degradation genes like *GWD* has been conducted. Although this strategy has been successful, the plant growth may have been inhibited because of the suppressed starch breakdown and the reduced carbon availability in the dark. Indeed, the *Arabidopsis sex1* mutant which was deficient in the *GWD* gene showed a strong growth suppression phenotype (Lloyd et al. 2005). Recently, the transient RNAi of the *GWD* gene was applied to *Arabidopsis*, indicating that the transient RNAi lines showed higher starch contents than the control plants without significant reductions of growth (Weise et al. 2012). It is also necessary, however, to develop simple methods to promote starch production in plants until such genetic

engineering can be widely applied to forage crops and biomass plants. The MeJA application did not result in growth retardation in *Arabidopsis* (Fig. 2c), possibly because MeJA, which did not affect the starch breakdown, did not suppress the carbon flow from starch. MeJA is a promising reagent which increases the starch content of forage crops and biomass plants to be used for energy. Considering that MeJA is a member of chemical groups related to fatty acids, more potent enhancers for starch production may be found among related compounds in the future. Further studies that screen out such compounds are needed to establish chemically controlled methods of starch production in plants.

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Figure legends

Figure 1 Effect of MeJA on starch accumulation in the areal part of *Arabidopsis*. (a) Starch staining. Plants were decolorized in hot 80% (v/v) ethanol and stained with iodine solution. Bars represent 1 cm. (b) Starch contents at the end of the light period. Values and bars represent means ± SD (n = 5). *Significant difference ($p < 0.05$) in comparison to control (0 μM MeJA) determined by Student's t-test. (c) Diurnal change of starch accumulation. Open and closed circles represent the control (0 μM MeJA) and treatment (500 μM MeJA), respectively. Values and bars represent means ± SD (n = 3). *Significant difference ($p < 0.05$) in comparison to control determined by Student's t-test at each time point. The white and gray areas correspond to the light and dark periods, respectively.

Figure 2 Effects of MeJA on starch content, anthocyanin accumulation, and fresh weight of the areal part of *Arabidopsis*. Starch content (a), anthocyanin accumulation (b) and fresh weight (c) are shown. MeJA was applied to *Arabidopsis* at the start of the light period (t0). Plants were harvested at the end of the light period. Values and bars represent means ± SD (n = 3). Open and closed circles represent control (0 μM MeJA) and treatment (500 μM MeJA), respectively. Gray and white bars above graph (a) indicate the dark and light periods, respectively. *Significant difference ($p < 0.05$) in comparison to control determined by Student's t-test at each time point.

Figure 3 Effects of MeJA on the expressions of starch metabolism-related genes. Transcript accumulation of each gene was measured using RT-PCR. (a) Starch synthesis-related genes; (b) starch degradation-related genes; (c) positive (*VSP1*) and negative (*ACT2*) control genes. Gene names and AGI codes are shown. Expression level of the control (0 μM MeJA) at 0 hours in each gene is standardized. White and gray columns represent control and treatment (500 μM MeJA), respectively. Values and bars represent means ± SD (n = 3).

Figure 4 Effects of MeJA on starch accumulations in the areal parts of tobacco (a, b) and spinach (c, d). (a, c) Starch staining. Plants were decolorized in hot 80% (v/v) ethanol and stained with iodine solution. Bars represent 1 cm. (b, d) Starch contents. Values and bars represent means ± SD (n = 5). *Significant difference ($p < 0.05$) in comparison to control (0 μM MeJA) determined by Student's t-test.

Supplementary material legends

Supplementary Table 1 Primers of polymerase chain reaction (PCR) in the corresponding genes tested in Fig. 3.

Title: Enhancement of starch accumulation in plants by exogenously applied methyl jasmonate

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Journal: Plant Biotechnology Reports

Supplementary Figure 1 Effect of jasmonic acid (JA) on starch accumulation in *Arabidopsis* leaves. *Arabidopsis* (ecotype Columbia) plants were grown in 7-cm plastic pots filled with Peatban (Sakata Seed, Yokohama, Japan) in growth chambers with 100 μmol m^{-2} s^{-1} light under long-day conditions (16 h light/8 h dark cycle) at 23 ℃. The density of planting was three plants per pot. (±)-JA (Cayman Chemical, MI, USA) was dissolved in ethanol at the concentrations of 0 (ethanol only), 100, 250, 500, and 1000 mM, respectively. Ten micro liters of the corresponding JA solutions were added to water (9.99 mL). The resulted solutions (0, 100, 250, 500, and 1000 μM JA) were used for the JA application. The JA solutions were sprayed on the surface of the leaves of three-weeks-old plants with a hand-pump aerosol spray bottle (1 mL per pot) at the start of the light period. At the subsequent end of the light period, the rosette leaves were harvested and used for the following starch analysis. Fresh tissues were treated twice with 10 volumes of 80% (v/v) ethanol at 80°C for 20 min. The ethanol insoluble residue was extracted by an equal volume of 0.4 M KOH at 80°C for 60 min. After the extract was neutralized, soluble starch was digested by 10 U α-amylase and 7 U amyloglucosidase. Glucose formation was determined by a glucose oxidase- and peroxidase-based enzyme assay. Starch content was calculated based on the released glucose. Values and bars represent means ± SD (n = 5). *Significant difference ($p < 0.05$) in comparison to control (0 μM JA) determined by Student's *t*-test.

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Figure

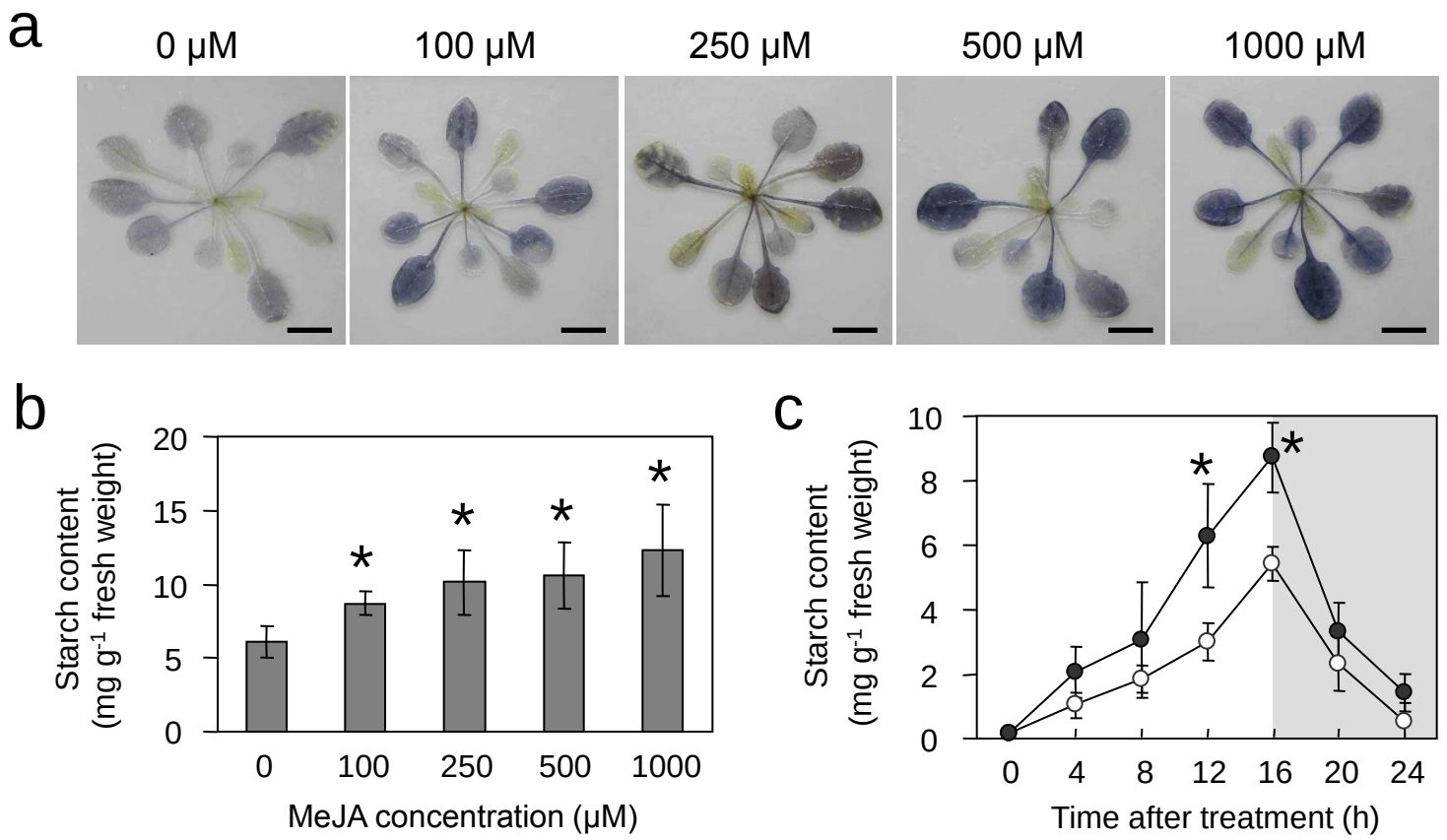

Fig. 1 Takahashi and Hara

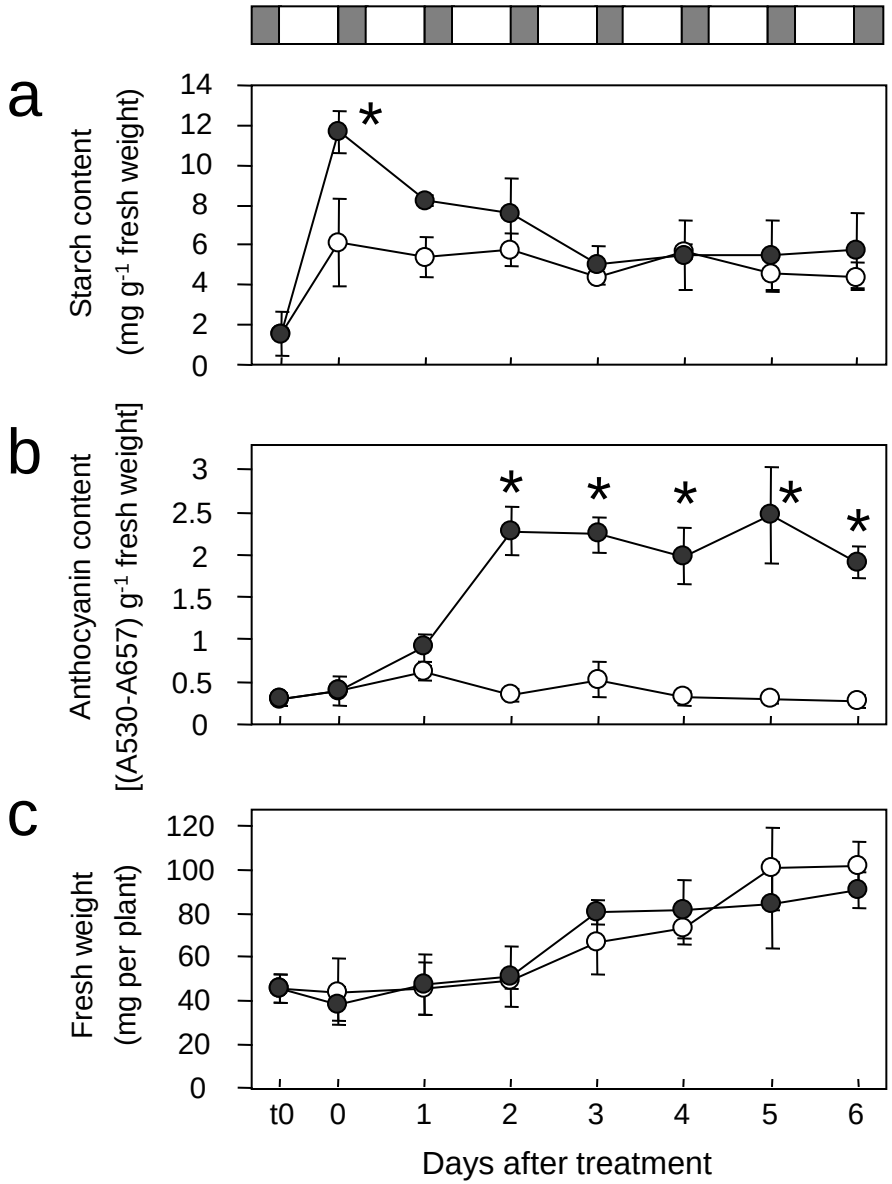

Fig. 2 Takahashi and Hara

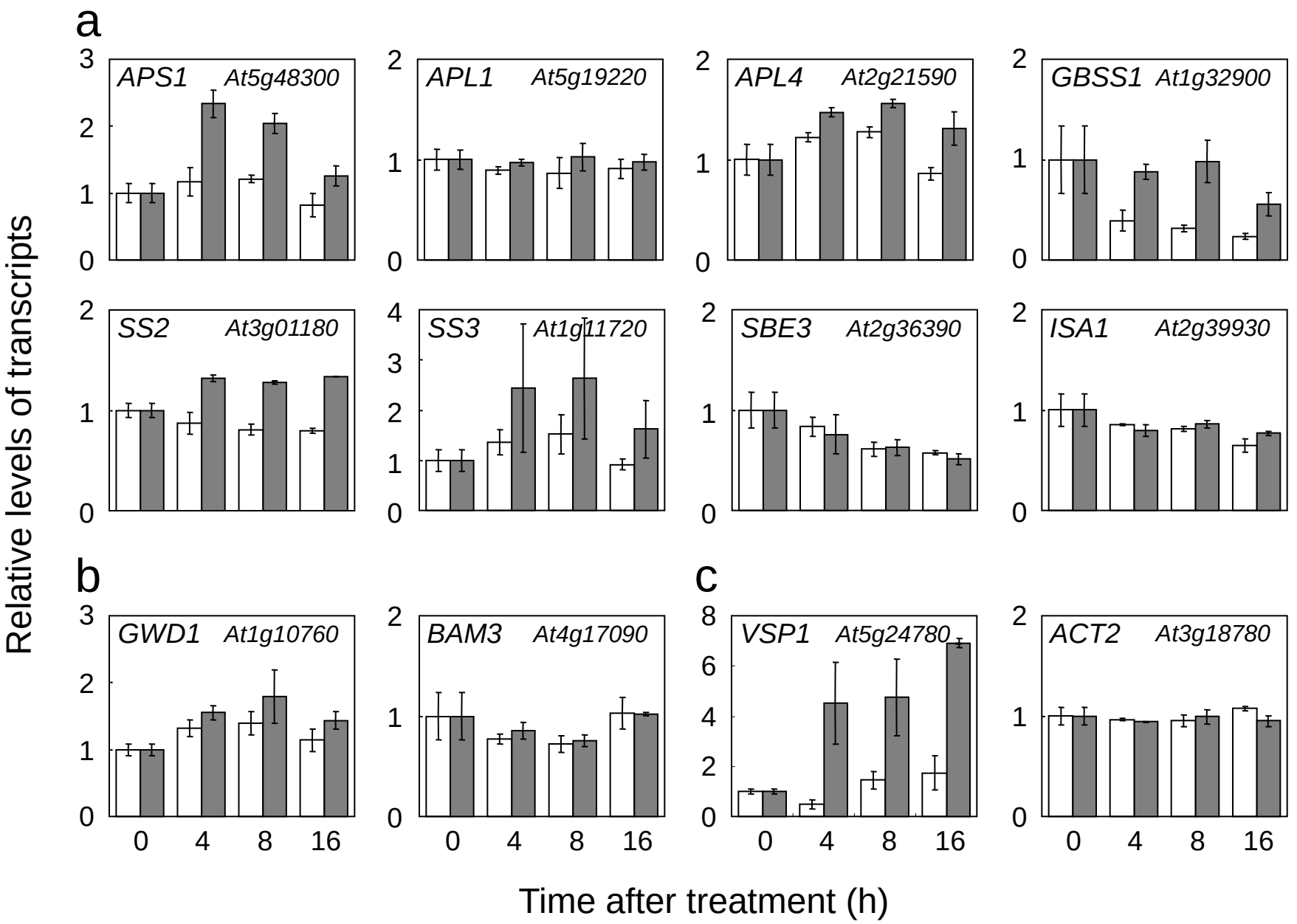

Fig. 3 Takahashi and Hara

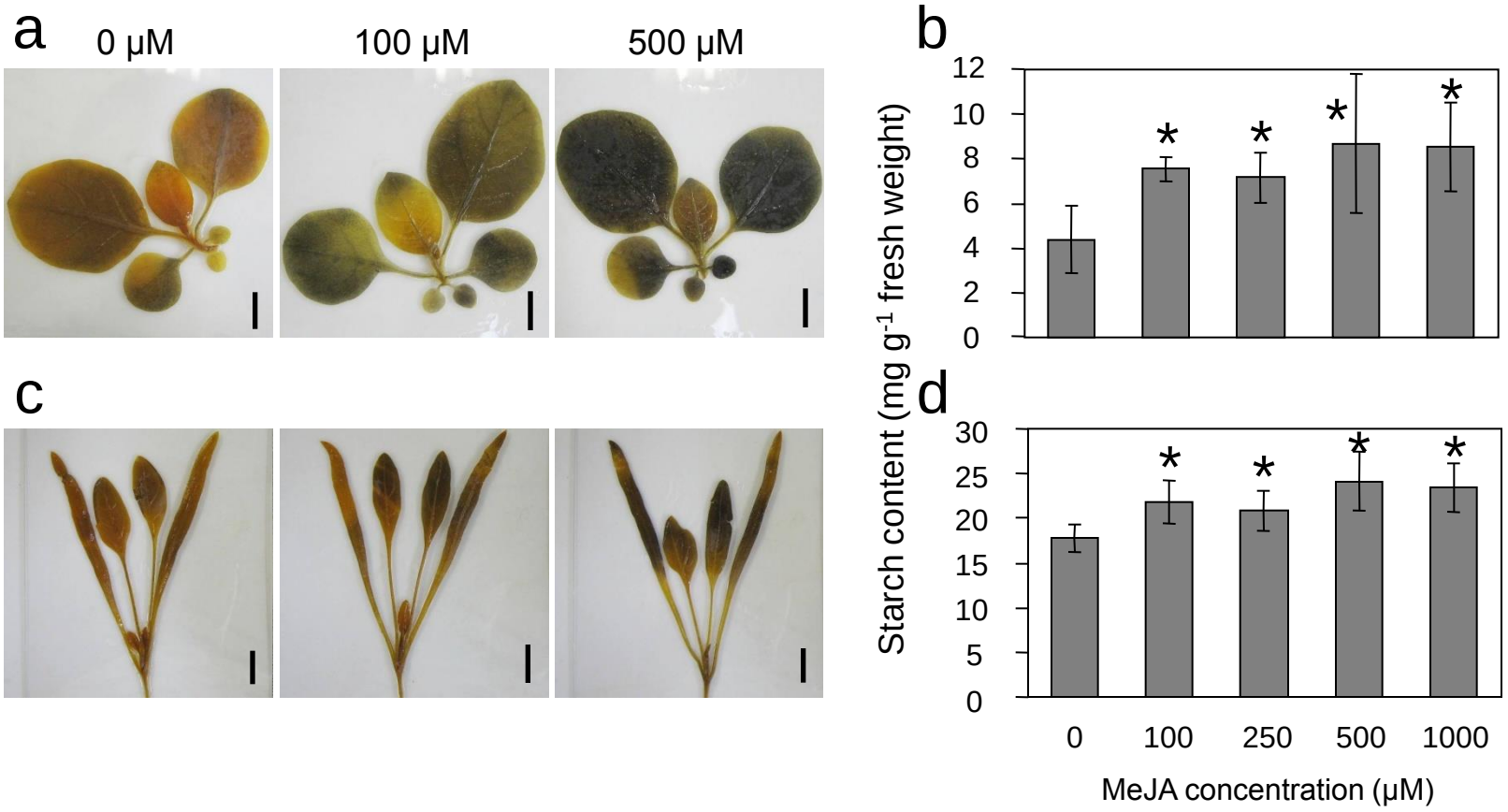

Fig. 4 Takahashi and Hara

Supplementary Material

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