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POTENTIAL HERBICIDE TARGET, 7-KETO-8-AMINOPELARGONIC ACID SYNTHASE

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ABSTRACT

The validation of potential herbicide target, 7-keto-8-aminopelargonic acid synthase (KAPAS) in the early step of biotin biosynthesis pathway, was performed in vitro and in vivo with lead chemical triphenyltin acetate (TPTA). KAPAS activity was completely inhibited by TPTA with an IC_{50} of 19.85 µM. 40-day-old Arabidopsis thaliana plants were killed with foliar treatment of 125 g/ha TPTA under the greenhouse conditions. The germination of A. thaliana seeds was also completely inhibited with 62.5 µM TPTA, but it was rescued by 85–92% with the supplement of biotin biosynthesis intermediates such as 0.5 mM of biotin, dethiobiotin, and 7, 8-diaminopelargonic acid, but not by 7-keto-8-aminopelargonic acid (KAPA). However, additional supplement of 0.5 mM S-adenosyl-L-methionine (SAM) with 0.5 mM KAPA rescued up to 91% of the germination previously inhibited by the 50 µM TPTA. Also, biotin supplements alleviated the growth inhibition of 40-day-old A. thaliana plant. Foliar application of TPTA induced 8-fold higher substrate (L-alanine) accumulation in the treated A. thaliana plants. RNA expression for KAPAS transcripts were much low in leaf tissue treated with TPTA. With these results, we report that SAM is an essential donor of amino groups for synthesis of the biotin precursor KAPA to 7, 8-diaminopelargonic acid (DAPA) synthesis in plants that KAPAS is a potential herbicidal target site in the biotin biosynthesis pathway, and that TPTA might be one of the potential non-comparative KAPAS inhibitors.

Key words: Biotin, KAPAS, SAM, target, TPTA.

INTRODUCTION

We have described the effects of expressing anti-sense RNA of cloned plant genes encoding for potential herbicide target enzyme 7-keto-8-aminopelargonic acid synthase (EC 2.3.1.47, KAPAS, also known as 8-amino-7-oxononanoate synthase) in stably transformed transgenic test plants (Hwang *et al*., 2003). Individual biotin auxotrophs for KAPA synthase, transformed with anti-sense *Arabidopsis thaliana* KAPAS (*AtKAPAS*) construct, exhibited

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considerable phenotypic alterations such as growth inhibition, severe growth retardation, yellow–green cotyledons and leaves as well as lethal phenotype (Fig. 1). These results suggest that the anti-sense disruption of *AtKAPAS* gene causes lethality in the early stage of plant development. 7-Keto-8-aminopelargonate synthase is a pyridoxal 5'-phosphatedependent enzyme which catalyzes the decarboxylative condensation of L-alanine with pimeloyl-CoA in a stereospecific manner to form KAPA, coenzyme A, and carbon dioxide in the first committed step of biotin biosynthesis. Perhaps the most important role of biotin is in the carboxylation of acetyl-CoA to give malonyl-CoA, which is the first step in fatty-acid biosynthesis. Since fatty-acid synthesis is essential for the growth and development of most organisms, biotin is thus an essential nutrient for plants and animals. Plants, micro-organisms, and some fungi biosynthesize their own biotin, while animals necessarily require trace amounts of the vitamin in their diet. Therefore, inhibition of the enzymes involved in the biotin biosynthesis pathway can cause irreparable damage to plants, and for this reason, such enzymes can be useful targets for the rational design of inhibitors in the hopes of finding new herbicides (Webster *et al*., 2000; Nudelman *et al*., 2004).

The aim of our investigation is to confirm that the genetic validation of KAPAS as a potential herbicide target enzyme, and chemical validation of TPTA as a lead compound for the potential KAPAS inhibiting herbicide derivatives *in vitro* and *in vivo*.

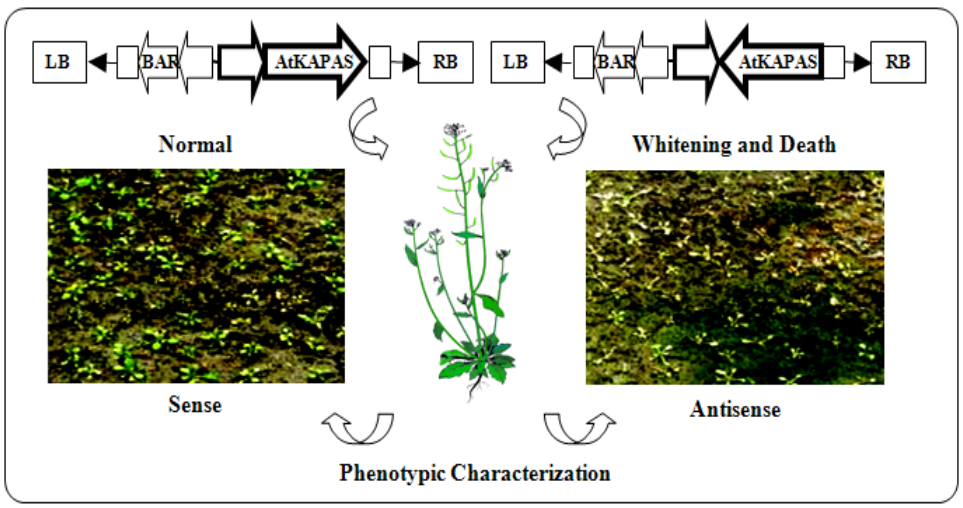

Figure 1. Sense and anti-sense expression of target gene in *Arabidopsis.*

MATERIALS AND METHODS

Cloning and Expression of *AtKAPAS*

Double-stranded cDNA was constructed from 5 µg of poly(A)+mRNA with the Time Saver cDNA synthesis kit (Pharmacia, Piscataway, NJ, USA), using Oligo(dT)18 as a primer. By performing PCR (polymerase chain reaction) with the two primers (KAPAFB, 5'-CAAAAAGAATTCGACGACGACGACAAGATGGCGGATCATTCGTGGGATAAA -3' and KAPARH, 5'-GTGCACCTCGAGTTATAATTTGGGAAA TAGAAAGGA-3'), the full-length AtKAPAS cDNA was amplified and isolated from *A. thaliana* cDNA library prepared. The resulting PCR fragment was digested with *Eco*RI and *Xho*I, and cloned into MBP (maltose binding protein) fusion vector (Bioprogen Co., Ltd., Korea) to generate construct pEMBPek-KAPAS (Fig. 2). *Escherichia coli* BL21-Gold(DE) (Stratagene, USA) was transformed with expression vector pEMBPek-KAPAS and then cultured in LB (Luria–Bertani broth, USB, USA) medium containing 100 µg/ml of ampicillin at 37°C (150 rpm) until the value of OD_{600} reached 0.6. In order to induce the expression of the target protein in *E. coli* cells, isopropyl-D-thiogalactoside was added to the suspension at a final conc. of 1 mM, and further cultured for 3h.

Substrate Synthesis and Enzyme Assay *in Vitro*

Pimeloyl CoA was synthesized according to the method of Ploux and Marquet (1992). TPTA was purchased from Sigma (USA) and used as a KAPAS-inhibitor. KAPAS activity was determined according to the method of Webster *et al* (2000). The procedure was the same apart from the reaction volume of 250 µl instead of 1 ml. The KAPAS concentration in all analysis was 10 µM in 20 mM potassium phosphate (pH 7.5) and the concentrations of TPTA were 3.125, 6.25, 12.5, 25, 50, and 100 µM. Reference cuvettes contained all other compounds except inhibitor.

Herbicidal Activity under Greenhouse Condition

Seeds of *A. thaliana* were sown in plastic pots (24 cm^2 surface area) filled with artificial nursery soil (Boo-Nong Soil, Seoul, Korea), and the plants were grown to the required growth stage for TPTA application in a greenhouse maintained at 25–30°C during the day and 20–25°C at night. The plants were TPTA treated (16, 32, 62.5, 125, 250, and 500 g/ha with laboratory spray gun (spray volume of 1,000 l/ha) 40 days after seeding. The herbicidal spectrum of TPTA was investigated in 10 weed species (*Sorghum bicolor*, *Echinochloa crus-galli*, *Agropyron smithii*, *Digitaria sanguinalis*, *Panicum dichotomiflorum*, *Solanum nigrum*, *Aeschynomene indica*, *Abutilon avicennae*, *Xanthium strumarium*, *Calystegia japonica*) with foliar application of 0.25, 0.5, 1, 2, and 4 kg/ha using a laboratory spray gun two weeks after sowing (in plastic pot (350 cm^2 surface area filled with

upland soil). Visual injury was determined at 2 weeks after application with a scale of 0 (no injury) to 100 (complete death).

Rescue of Seed Germination and Plant Growth

Germination test: Seeds of *A. thaliana* were germinated in 55 mm plastic Petri-dish lined with one-layer filter paper (Advantec No. 2). About 1 ml of each TPTA solution dissolved in absolute acetone with various concentrations of 0, 0.0063, 0.0125, and 0.025 mM was spread evenly onto the filter paper (Ø 5 cm), respectively and allowed to dry in a laboratory fume hood. After that, 1 ml of distilled water with or without supplement of 0.5 mM biotin (Sigma, USA), dethiobiotin (Sigma, USA), 7,8-diaminopelargonic acid (DAPA, Synthesis), and KAPA (TRC, Inc., Canada) was added, and 30-seeds were placed onto the filter paper in Petri-dish. Each Petri-dish was sealed with laboratory film and held in an incubator at 25^{o}C, 14/10 h (Light/Dark). Plant growth test: *A. thaliana* of 40-day-old plants as reported above were used. 1 mM biotin was supplemented by foliar laboratory spray gun with spray volume of 5,000 L/ha at each 1 or 2 days prior to 100 g/ha TPTA application. At 5 days after TPTA application, plant leaves were harvested and chlorophyll content was determined following the method reported by Hiscox and Israelstam (1979). Chlorophyll content was calculated following the equation used by Arnon (1949).

L-Alanine accumulation

Alanine was determined using a detection system of copper complex with L-alanine described by Nakao *et al* (1986) with some modifications (Weinstein, 1984; Lin and Wu, 2005). Forty-day-old *Arabidopsis* plants grown as above were treated with TPTA (200 g/ha) by foliar application with laboratory spray. Plant leaves were harvested at 3 days after TPTA application. The copper complex of L-alanine was determined by the optical density at 620 nm of the supernatant (200 µl) using a microplate spectrophotometer (Benchmark Plus, Bio-Rad, USA). The concentration of L-alanine was determined by standard curve prepared from the same method with various concentrations of L-alanine. The standard curve was calculated as $Y = 0.4695X + 0.0146$, $r^2 = 0.9993$.

RNA Isolation and RT-PCR Analysis

RT-PCR (Reverse transcription-polymerase chain reaction) amplifications were performed with an iCycler™ Thermal Cycler (BIO-RAD, http://www.bio-rad.com/), according to the manufacturer's instructions. RNA was prepared from various tissues of *Arabidopsis* that had been immediately frozen in liquid nitrogen under RNase-free conditions. The RNA was isolated with the Qiagen RNeasy Plant Mini Kit (Qiagen, http://www.giagen.com/) for subsequent reverse transcription reactions. First-strand cDNA was synthesized with 1 µg of

total RNA using the Oligo(dT)12–18 primer and the SuperScript™ III Reverse Transcriptase, (Invitrogen, http://www.invitrogen.com/), following the manufacturer's instructions. One microliter of cDNA was used for PCR reactions. The PCR conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 26 cycles of 94°C for 2 min, 55°C for 40 s and 72°C for 1 min. KAPAS-specific primers for RT-PCR were: KAPAS-F, 5'-GCTGAACGACAAGGA AATGTTG-3'; KAPAS-R, 50-GAGTGGCTGTGTTGTCAAAG-30. Primers for amplification of reference gene, tubulin was: TUB-F, 5'-CTCAAGAGGTTCTCA GCAGTA-3'; TUB-R, 5'-TCACCTTCTTCATCC GCAGTT-3'.

RESULTS

The *AtKAPAS* cDNA was cloned into MBP fusion vector to generate the *E. coli* expression construct pEMBPek-KAPAS. SDS–PAGE analysis revealed that *E. coli* transformed with MBP fusion vector showed the expression of a very strongly induced fusion protein of ca. 98.2 kDa, which consisted of the *AtKAPAS* protein of 51.3 kDa and the maltose binding peptide MBP affinity tag of 46.9 kDa. For purification of *AtKAPAS* protein, the lysates from IPTG-induced *E. coli* containing pCKAPA as well as from *E. coli* harboring control vector MBP fusion vector were loaded onto maltose affinity column (1.1 cm x 30 cm, Millipore, USA). Elutes of *E. coli*-expressed *AtKAPAS* protein contained the induced fusion protein of ca. 98.2 kDa while those of *E. coli* control did not. *AtKAPAS* protein was expressed in *E. coli* at a very high level, and a significant portion of these proteins was soluble, and their affinity-purified preparations contained a single major polypeptide (Figure 2). The dose-dependent *in vitro* inhibition of KAPAS activity by TPTA was examined and the IC_{50} was calculated as 19.85 µM. The foliar-treatment of 16, 32, 62.5, 125, 250, and 500 g/ha TPTA to the 40-day-old *A. thaliana* plants caused visual injury of 8.3, 20, 47, 90, 97, and 100%, respectively, and herbicidal activity increased with time. The application rate of more than 125 g/ha caused almost complete death at 1 week after application. The main symptoms were tissue collapse and desiccation. Symptoms began to appear within several hours after treatment, and the application region in the leaf was desiccated at 1 day after treatment of higher than 250 g/ha. Foliar application of TPTA to 10 weed species showed good herbicidal activities. The most sensitive species was *Xanthium strumarium* which was completely dead at 250 g/ha of TPTA foliar application. *Abutilon avicennae*, *Calystegia japonica*, and *Aeschynomene indica* were also controlled by 500 g/ha of TPTA foliar application (Table 1). Grass weeds such as *Sorghum bicolor*, *Echinochloa crus-galli*, *Agropyron smithii*, *Digitaria sanguinalis*, and *Panicum dichotomiflorum* were tolerant to TPTA foliar application compared to the broad-leaf weeds.

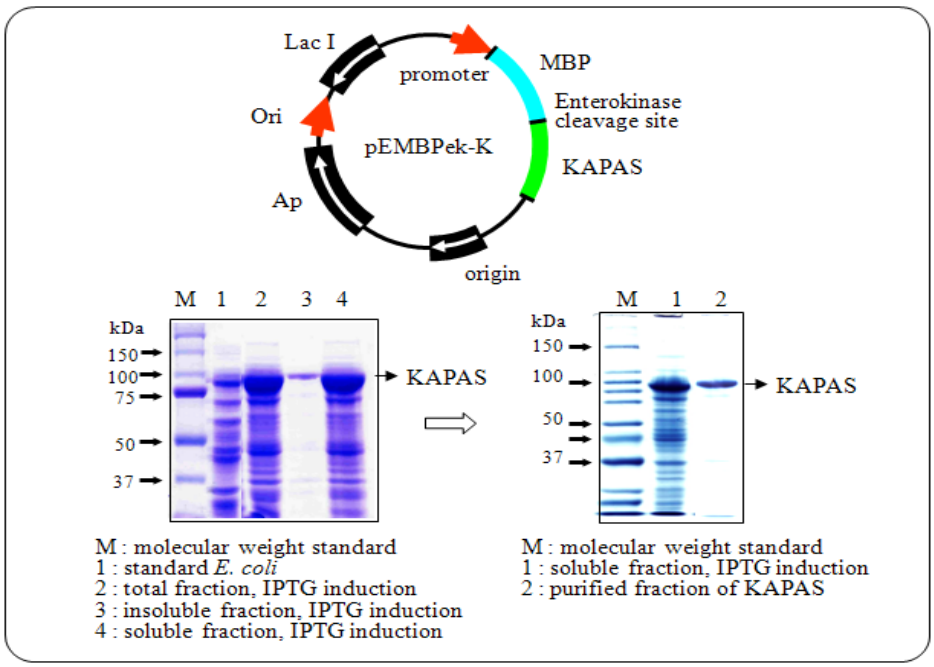

Figure. 2. KAPAS over expression and purification from transgenic *E. coli*.

Table 1. Herbicidal activity of triphenyltin acetate (TPTA) on several weed species under greenhouse conditions.

Rate (kg/ha)	Control value (%)									
	SORBI	ECHCG	AGRSM	DIGSA	PANDI	SOLNI	AESIN	ABUTH	XANSI	CAGEH
0.25	30	40	0	30	50	30	70 C	40 C	100	30
0.5	40	40	0	50	60	80	95	100	100	100
1	40	40	0	50	60	80	95	100	100	100
2	40 C	60	60	50	80	100	100	100	100	100
4	70 CI	70 BC	50 BC	60 N	100	100	100	100	100	100

SORBI, *Sorghum bicolor*; ECHCG, *Echinochloa crus-galli*; AGRSM, *Agropyron smithii*; DIGSA, *Digitaria sanguinalis*; PANDI, *Panicum dichotomiflorum*; SOLNI, *Solanum nigrum*; AESIN, *Aeschynomene indica*; ABUTH, *Abutilon avicennae*; XANSI, *Xanthium strumarium*; CAGEH, *Calystegia japonica*. Pre, pre-emergence application; Post, post-emergence application. Description of footnotes: B, stunting; C, desiccation; I, chlorosis or abnormal color of plant; N, bleaching (lack of pigmentation). Visual injury was determined at 2 weeks after application with a scale of 0 (no injury) to 100 (complete death).

The germination of *A. thaliana* seeds was almost completely inhibited by 0.05 mM TPTA. Also, TPTA at > 0.125 mM significantly

reduced the plant growth at early stage after seed germination. However, the inhibited germination by 0.05 mM TPTA was recovered by 85–92% with the supplement of 0.5 mM biotin, dethiobiotin, or DAPA, but not by KAPA, one of the biotin biosynthesis intermediates (Fig. 3). Additional supplement of 0.5 mM SAM with 0.5 mM KAPA increased up to 91% of the germination inhibited by 0.05 mM TPTA (Fig. 4).

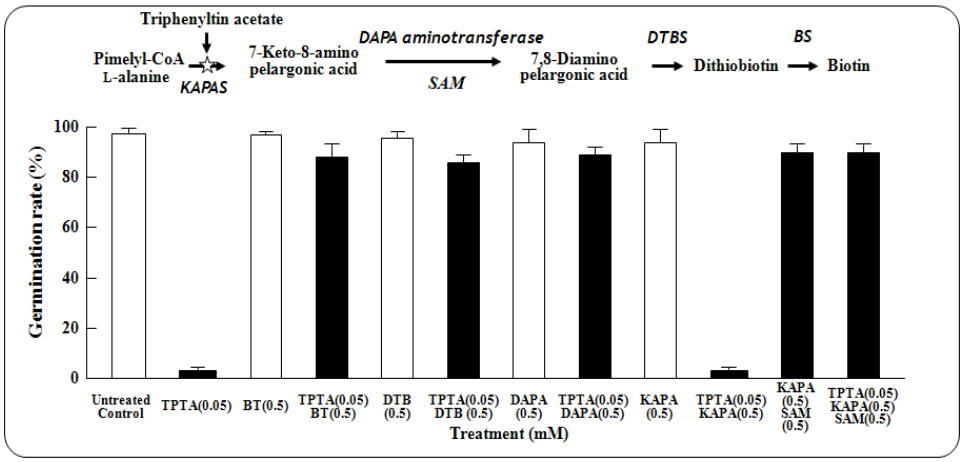

Figure 3. Reversal of *A. thaliana* seed germination with biotin biosynthesis intermediates supplement. KAPAS, 7-keto-8-aminopelargonic acid synthase; DAPA, 7,8-diamino pelargonic acid; DTBS, dithiobiotin synthase; BS, biotin synthase; TPTA, Triphenyltin acetate; BT, Biotin; DTB, dithiobiotin; SAM, *S*-adenosyl-L-methionine.

Chlorophyll content in *A. thaliana* plant treated TPTA without biotin pretreatment was 10.7 mg/l, and that in untreated control *A. thaliana* plant was 20.5 mg/l. In contrast, the amount of chlorophyll extracted from the *A. thaliana* plant treated TPTA at 1 and 2 days after biotin pretreatment was 19.5 and 19.8 mg/l, respectively. It is clear that chlorophyll loss of *A. thaliana* plant treated with TPTA was recovered by biotin pretreatment at 1 & 2 days before TPTA application (Fig. 5).

Consequently, biotin pretreatment reversed the growth inhibition of *A. thaliana* plant by TPTA at the similar extent to the untreated control plants. 1.28 mM of L-alanine was detected from *A. thaliana* plants treated with 200 g/ha of TPTA, in contrast to 0.16 mM of L-alanine from untreated plants. The TPTA application induced 8-fold greater L-alanine accumulation in the plants (Fig. 6).

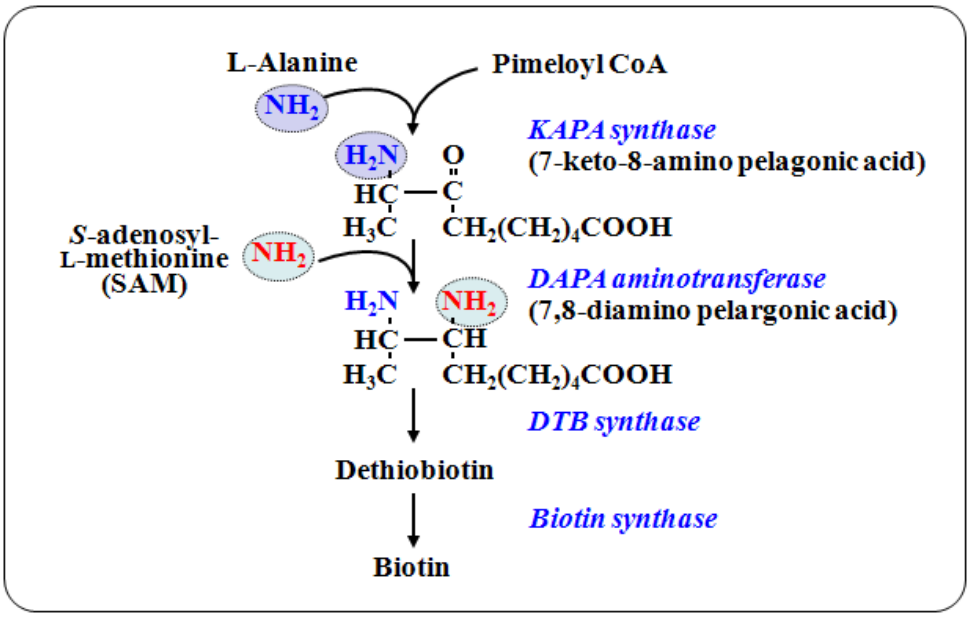

Figure 4. Proposed pathway of biotin biosynthesis containing SAM as amino group donor.

To expand our understanding on the role of TPTA, KAPAS gene expression in the root, leaf, stem, and whole plant of *A. thaliana* was analyzed by RT-PCR at 1 day after treatment with or without 100 g/ha TPTA (Fig. 7). KAPAS was expressed in most tissues, with the highest levels either in stems or roots of the untreated plants. Tubulin was used as a reference for gene expression in *A. thaliana*. However, RNA expression of KAPAS band was much fainter in the lane representing leaf tissue of TPTA (+) plants. Also, less RNA appears to the tublin band than in the other lane. This result implies that TPTA treatment reduces KAPAS expression in the leaf within 1 day of treatment like *bio1* mutants.

DISCUSSION

As a number of enzymes in the metabolic pathways of plants are essential for growth and development, those can be utilized as potential herbicide targets. We performed molecular genetics dissection using reverse genetics of anti-sense approach to identify *AtKAPAS* gene encoding KAPA synthase in the pathway of biotin biosynthesis and to characterize the phenotypic consequences of loss-of-function mutations. Many researchers have investigated the KAPAS in micro-organisms and the most of these reports were focused on the

biosynthesis in micro-organisms (Eisenberg and Star, 1968), purification and characterization (Ploux and Marquet, 1992; Stoner and Eisenberg, 1975), crystal structure (Alexeev *et al*., 1998; Kack *et al*., 1999), binding and kinetics (Ploux *et al.,* 1999), point mutation (Andrew *et al.,* 2002) and stereospecificity (Vikrant *et al.,* 2006).

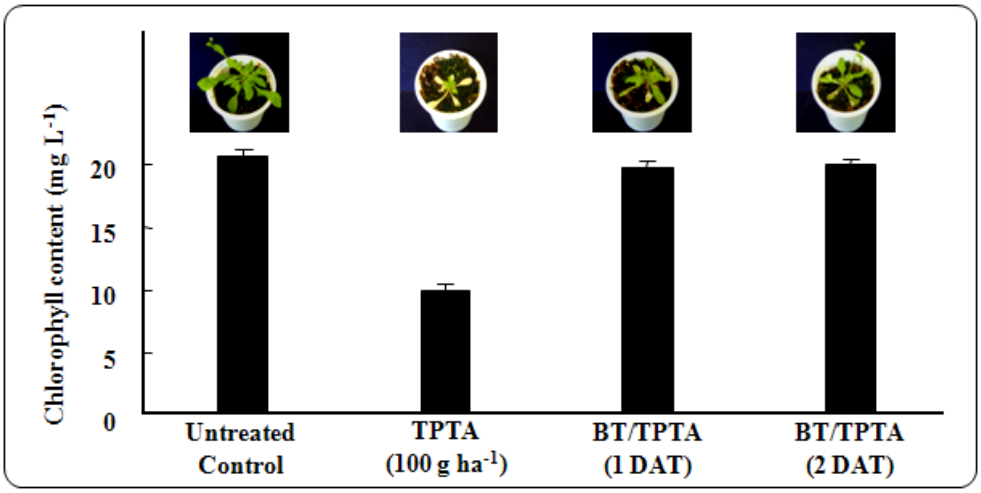

Figure 5. Reversal of *A. thaliana* growth inhibition with biotin supplement. TPTA, Triphenyltin acetate; BT, Biotin; BT/TPTA, BT treatment followed by TPTA; DAT, day after treatment.

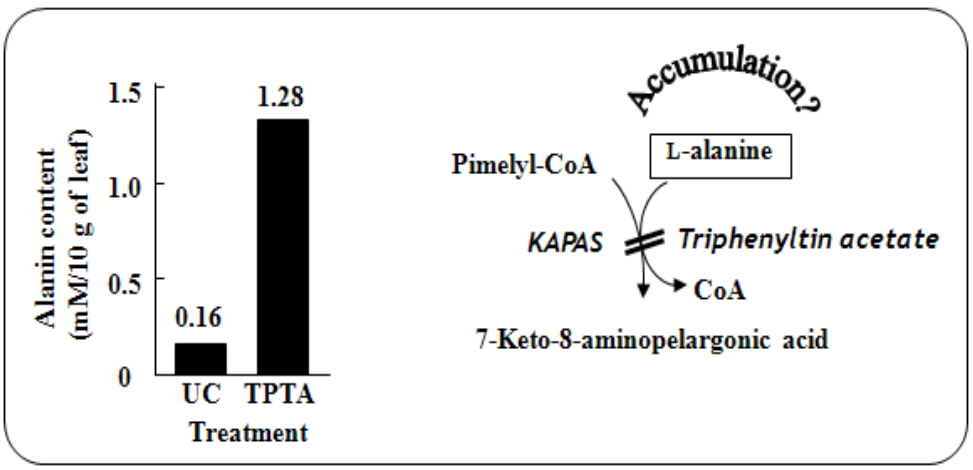

Figure 6. L-alanin accumulation in *A. thaliana* plants treated with triphenyltin acetate. KAPAS, 7-Keto-8-aminopelargonic acid synthase; UC, untreated control; TPTA, triphenyltin acetate.

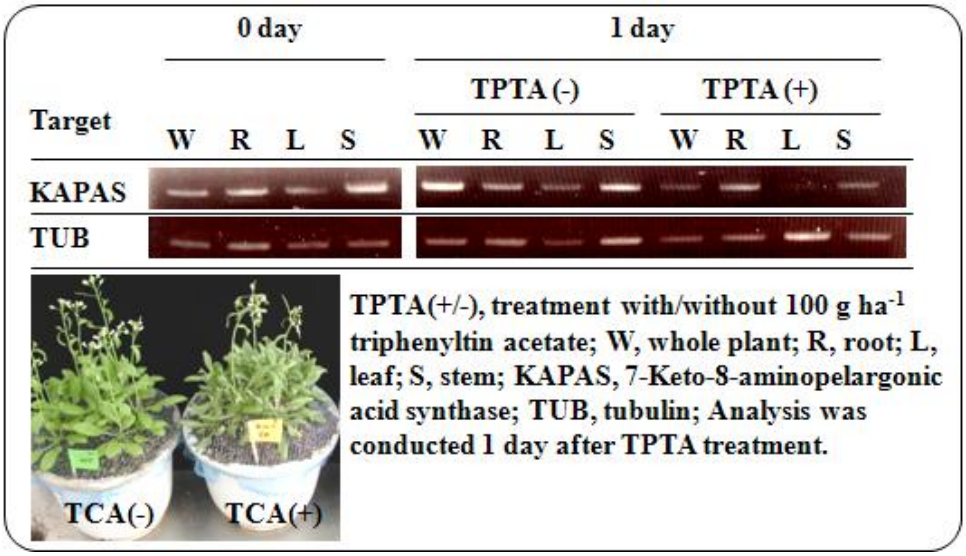

Figure 7. Semi-quantitative RT-PCR analysis of KAPAS gene expression in *A. thaliana* plants treated with or without 100 g/ha triphenyltin acetate.

Among them, Ploux *et al.* (1999) reported that the KAPAS catalyzes the first committed step of biotin biosynthesis in micro-organisms and plants, and suggested that the inhibitors of this pathway might lead to antifungal or herbicide agents. Webster *et al.* (2000) also reported that biotin is an essential enzyme cofactor for carboxylase and transcarboxylase reactions. The biosynthesis of biotin appears to follow similar pathways in both plants and micro-organisms, and thus, inhibition of the enzymes involved in the pathway is potentially an attractive target for both herbicide and antibiotics development.

Herbicidal symptoms after foliar treatment with TPTA were similar to herbicides targeting on the inhibition of fatty-acid biosynthesis in grasses, leading to death of the susceptible plants. In this point of view, the mode of action of TPTA might be correlated with the fatty-acid biosynthesis because the most important role of biotin is carboxylation of acetyl-CoA to give malonyl-CoA, which is the first step in fatty-acid biosynthesis. Biotin is an essential vitamin and acts as cofactor for a number of enzymes involved in facilitation of CO_2 transfer during carboxylation, decarboxylation, and transcarboxylation reactions that are related to fatty acid and carbohydrate metabolism. These biotin-dependent carboxylases in plants include cytosolic acetyl-CoA carboxylase, chloroplastic geranyl-CoA and acetyl-CoA carboxylases, and mitochondrial methylcrotonoyl-CoA carboxylase.

This complex contribution of biotin and biotin-mediated reactions in the plant cell implies an intracellular trafficking of biotin and precursors, thus requiring transport mechanisms. These transport steps include transfer of an intermediate, KAPA, DAPA, or dethiobiotin, between the cytosol and mitochondria was demonstrated by Pinon *et al.* (2005).

With these results, we report that SAM is an essential donor of amino groups for synthesis of the biotin precursor KAPA to 7,8-diaminopelargonic acid (DAPA) synthesis in plants, that KAPAS is a potential herbicidal target site in the biotin biosynthesis pathway, and that TPTA might be one of the potential KAPAS inhibitors.

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