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7-Keto-8-Aminopelargonic Acid Synthase as a Potential Herbicide Target

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1. Introduction

Agrochemicals are compounds that selectively kill or arrest the growth of pests and weeds. They have played a significant role in agricultural production that provided for about 600 million people during the past 50 years. And also, the increasing world population seems to be a major driving force for the need to enhance the output of food production per area (Joseph, 2004). The agrochemical industry has been very successful in developing new herbicides. New chemicals with improved properties, especially providing significantly reduced application rates, and often new modes-of-action have been discovered, developed and launched for diverse crops. This success has positively influenced agriculture as a whole. However, in these days the introduction of new herbicides with either a new mode of action or novel chemical classes has lingered. After launch of sulcotrione, a HPPD herbicide at 1991, any herbicide with new mode-of-action has not been commercialized in Europe, while there were 10 new modes-of-action commercialized between 1970 and 1985 and five new ones between 1986 and 1991 (Schulte, 2004; Rüegg, 2007).

Are there still opportunities for new herbicides, and what are the main search targets? Is there still an incentive to invest into herbicide research? Many factors adding complexity are agronomic, structural and technological changes, including the introduction of herbicide-tolerant crops, and the high costs of development for new active ingredients, mainly due to increasing regulatory requirements. In the light of increasing weed resistance to widely used herbicides, securing diversity in agronomy as well as weed management is a key to efficient crop production in future. Further problems to be addressed are the expectations regarding weed shifts and/or the occurrence of (new) weed problems, due to the introduction of new weed species by global travel or international transport of goods. Will certain plants profit from climatic changes like global warming? The increase in the global population has already led to an intensification of crop production and this must continue in order to secure world food supply. In order to secure crop yields, chemical solutions for weed management will continue to be the preferable choice for the predictable future because apparent alternatives are not in sight. In order to support this objective, new herbicides, preferably with new modes-of-action, need to be discovered and developed.

Until recently, the first step in agrochemical discovery was to take a collection of chemicals, apply each to a small population of representative pests, and assess their efficacy by visual inspection. This approach, sometimes impolitely called 'spray and pray', has its strengths

(Wolfgang et al. 2004, Manjula et al. 2010). It takes advantage of the obvious fact that it is easier to wreck a system than to fix it. The testing of chemicals for efficacy on whole plants is direct and integrates several important attributes that are needed for a crop protecting, including its uptake, transport, metabolism and ability to inhibit an important target protein. This approach does not require any detailed knowledge about the biochemical or cellular target, which was important in an era when our understanding of biology was poor. Following the initial identification of a lead chemical, intensive research and testing followed to optimize its structure to understand its action, and provide data on its environmental compatibility. However, the traditional approach depended on serendipitously discovering a chemical structure that could enter the pest, be transported within it, inhibit a key target, get away from detoxification, and also be modified to allow it to fulfill increasing-regulatory criteria with respect to environmental compatibility. Testing chemicals on whole organisms is logistically demanding, especially if the organisms are relatively large, relatively large amounts of chemicals required, so the ability of chemists to synthesize sufficient quantities of new structures becomes another serious limitation on the number of chemicals that can be tested (Wolfgang et al. 2004).

Currently, studies of environmental compatibility have become an increasingly large part of the entire research effort because the market was progressively occupied by effective agrochemicals and because hurdles have been enlarged with respect to environmental compatibility. Typically, 10-12 years are necessary to develop a lead chemical structure into a market product. Since the discovery of the auxinic herbicides in the late 1940s, empirical screening has led to the commercialization of around 270 active ingredients, representing 17 modes of action (Ott et al. 2003). Of these herbicides, approximately 50% act on one of only three targets: acetolactate synthase, photosystem II, or protoporphyrinogen oxidase. In addition, 10 herbicides account for 45% of the total market value. Thus, the major herbicides on the market act on only a handful of targets, whereas it is quite evident that there are many more ways to kill a plant.

In the past 10 years, strategies for the first steps of herbicide discovery have switched from the testing of chemicals for efficacy on whole plants towards a target-orientated approach using *in-vitro* assays against molecular targets, it is obviously essential to choose appropriate targets. Therefore, target-directed high throughput screening (HTS) systems are implemented as additional tools in addition to greenhouse screening. This requires the identification of proteins whose inhibition will lead to the death or a severe growth arrest of the objective organism. Many different approaches have been developed to identify *bona fide* targets for *in-vitro* screening (Wolfgang et al. 2004, Manjula et al. 2010). Developments in functional genomics could aid the development of assay systems for the evaluation of chemicals for their suitability as lead structures in herbicide discovery (Ott et al. 2003).

2. How can we select *bona fide* target?

Most of the herbicides attack to the unique biochemistry of plants causing severe disruption of the plant metabolism. These are usually inhibitors of specific enzymes binding either at the active site of the enzyme or at some domain apart from the active site (Berg et al., 1999; Dayan et al. 2009). Among the strategies to identify suitable targets, one strategy was to assume that if an enzyme in a pathway or process is a target, then others in the same pathway or process might be too. The problems and limitations of this 'copy cat' approach have been nicely reviewed (Abell, 1996). Another strategy is to use literature survey to

identify 'key' or 'limiting' protein in the essential process that would catalyze irreversible reactions and is highly regulated. Third approach, a revolutionary tool in herbicide discovery, is to provide genetic evidence that the gene encodes the essential target protein (Wolfgang et al., 2004). An antisense technology was used to demonstrate that dehydroquinate dehydrase/ shikimate dehydrogenase constitutes an herbicide target (Freund et al., 2002). Genetic pre-validation of targets in a systematic manner started in the early 1990s, soon after routine methods for plant transformation were established. In its first phase, this approach was focused on specific pathways that were thought to be essential for the plant. The relevance of selected candidates was tested by partial inhibition of their activity using co-suppression or antisense strategies, which resulted in the variable inhibition of expression at the protein level. Typically, about 10% of the plants show a significant decrease in protein expression, with the extent of the decrease varying from 30~90% depending on the transgenic line. The inhibition of expression at the protein level can be quantified using measurements of enzyme activity in standardized conditions, and compared with the inhibition of growth and other phenotypical or biochemical changes in the plants. About 20% of the enzymes in these central pathways qualified as potential herbicide targets. Crucially, they would not have been reliably predicted by the traditional criteria for identifying 'key' regulated enzymes. Many highly regulated enzymes that catalyze irreversible reactions could be strongly inhibited without a significant impact on growth, whereas some of the experimentally validated targets were transporters or enzymes that catalyze readily reversible reactions. The accumulation of large amounts of sequence information from the late 1990s onwards, first as a result of expressed sequence tag (EST) sequencing and later from full-genome sequencing, made it possible to use unbiased and genome-wide strategies to identify targets. Nevertheless, the function of a large proportion of genes is either only vaguely annotated (around 50%) or completely unknown (more than 30%).
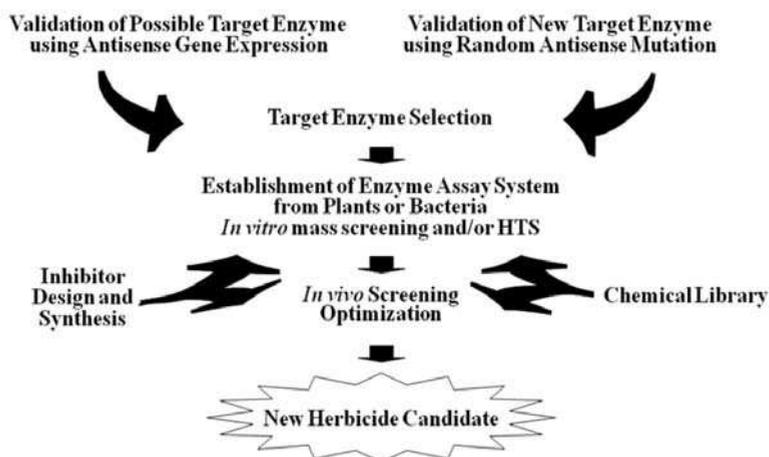

Fig. 1. Process of target search with antisense technology

Genetic approaches include studies of conditionally lethal bacterial and plant mutants and use of antisense technology (Fig. 1). In the absence of chemical leads with known sites of action, targets for validation may be selected by the following criteria: the target is essential to plants and, preferably, inhibition leads to multiple deleterious effects; the target is not

present in mammals; the target has low intracellular concentration, i.e., has potential for low use rates; and the proposed inhibitors of the target are synthetically accessible. Potent inhibition of the selected target may still not produce an effective herbicide. Studies of the uptake, translocation and metabolism of the inhibitor are needed to determine if the cause of poor *in vivo* performance is due to these factors or to an intrinsically poor target. Without full appreciation of each of these aspects of herbicide design, the chances for success with the target-site directed approach are reduced. Promising target enzymes were established as 4-hydroxyphenylpyruvate dioxygenase, adenylosuccinate synthetase, AMP deaminase, anthranilate   synthase, ascorbate peroxidase, asparagine synthetase, auxin transport, cytosolic glutamine   syhthetase, dihydro dipicolinate synthase, dihydrodipicolinate reductase, carboxypeptidase A, chloroplast NADH dehydrogenase, cinnamyl-alcohol dehydrogenase, geranylgeranyl diphosphate synthase, glutamate dehydrogenase, glutamate synthetase, glutamate-1-semialdehyde aminotransferase,   glutamate synthase, histidine biosynthesis, imidazoleglycerol phosphate dehydratase, isopropylmalate dehydrogenase, isopropylmalate isomerase, pheophorbidase, farnesyl transferase, *p-* hydroxyphenyl pyruvate dioxygenase, plasma membrane H+-ATPase, pyruvate orthophosphate dikinase, threonine dehydratase etc. (Kishore and Shah, 1988; Schloss and Aulabaugh, 1990; Abell et al., 1993; Rendina and Abell, 1994; Pillmoor et al., 1995; Abell, 1996; Kleier and Hsu, 1996; Subramanian et al., 1997; Bartley et al., 1999; Coulter, 1999; Cromartie et al., 1999; Ficarelli et al., 1999; Grossmann and Schiffer, 1999; Saari, 1999; Hwang et al., 2001).

This chapter, which focuses mainly on antisense technology, assesses progress being made and points to areas of research and new technologies regarding validation of the target KAPAS that have the potential to further increase the effectiveness of KAPAS inhibitor research. Successful design of novel herbicides based on the specific inhibition of selected enzyme targets requires careful consideration of the choice of the target, mechanism of the enzyme, design of potent inhibitors, delivery of the inhibitor to the target and metabolic fate of the inhibitor. Validated targets, those that produce phytotoxic effects upon partial inhibition, can be identified by genetic methods or by obtaining chemical leads. The aim of our investigation is to confirm that a particular enzyme chosen is indeed essential for a plant growth, and to validate the successful inhibition of the enzyme can lead to an herbicidal effect. Herein, we describe 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*.

3. Discovery of 7-keto-8-aminopelargonic acid synthase

In a pioneering pilot study (Jun et al., 2002), *Arabidopsis* antisense lines were created using randomly selected cDNAs. These lines were then scored for mutant phenotypes and analyzed genetically to exclude mutants that were clearly not caused by antisense inhibition of gene expression. At present, about 10,000 genes have been put through the entire process, including confirmation by independent retransformation, and 46 potential herbicide targets have been identified. These are genes whose partial inhibition leads to chlorosis, necrosis, and concomitant growth defects. They contain both known herbicide targets (e.g. glutamine synthetase) and genes for which antisense has already been reported to mimic herbicidal phenotypes (e.g. Rubisco and foredooming: NADP oxidoreductase) (Stitt et al., 1999; Palatnik et al., 2003).

Among them, we have already described expressing antisense RNA of cloned plant genes encoding for a 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; 2010). Individual biotin auxotrophs for KAPA synthase, transformed with antisense *A. thaliana* KAPAS (*AtKAPAS*) construct, exhibited considerable phenotypic alterations such as growth inhibition, severe growth retardation, yellow-green cotyledons and leaves as well as lethal phenotype (Fig. 1). We performed the database screening of *Arabidopsis* genome sequence with *bioF* sequence of *E. coli, B. subtilis,* and *B. sphaericus*. Through the analysis of cDNA isolated by PCR amplification, *AtKAPAS* gene (TAIR accession number 3443298) contained an open reading frame (ORF) of 1,410 base pairs encoding a putative protein of 469 amino acids with a predicted molecular mass of 51.3 kDa. *AtKAPAS* contains the domain of predicted aminotransferase class I and II in the C-terminal region, as well as the domain of putative plasma membrane spanning region.
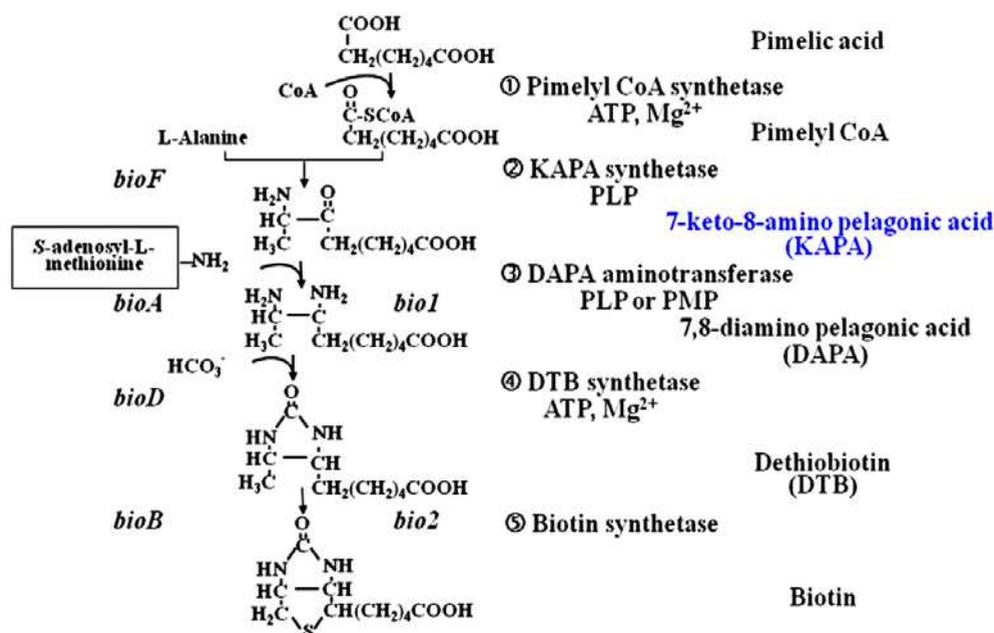

Fig. 2. Biosynthetic Pathway of Biotin in Microorganisms

Biotin is an essential vitamin and acts as a 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 (Dakshinamurti and Bhagavan, 1985; Alban et al., 2000). Bacteria, plants, and some fungi make their own biotin directly from endogenous biochemical intermediates, whereas other organisms such as most fungi and animals must obtain it from their surrounding environments. Therefore, the studies on the inhibition of the enzymes involved in the biotin pathway will potentially offer an attractive target for herbicide development. Since Eisenberg and Star (1968), Eisenberg and Stoner (1971) and Pai (1975) have first investigated on the biosynthetic pathway of biotin in *Escherichia coli* and *Bacillus subtilis* through biochemical studies including the analysis of auxotrophic mutants.

A number of researchers have widely investigated the biosynthetic pathway of biotin through combined biochemical and genetic studies in the bacteria (Ploux and Marquet, 1992; Alexeev et al., 1998; Huang et al., 1995). Also, there have been numerous studies on the related gene for biotin synthesis in a variety of other microorganisms (Zhang et al., 1994; Fleischmann et al., 1995; Bult et al., 1996). The ordinary pathway for biotin biosynthesis in microorganisms and plants is shown in Fig. 1 (Patton et al., 1998). Most steps in the pathway have been clearly investigated in the microorganism, particularly *E. coli* and *Bacillus* spp. *E. coli* and *B. subtilis* have only one *bio* cluster consisted of five genes: *bioABFCD* and six genes: *bioWAFDBI*, respectively (Bachman, 1990; Bower et al., 1996), whereas *B. sphearicus* has two separate clusters consisted of seven genes: *bioXWF* and *bioDAYB* (Gloeckler et al., 1990). In *E. coli*, *BirA* protein is known to act as a negative regulator for the expression of the biotin operon by interaction in a region between *bioA* and *bioB* (Barker and Campbell, 1981). The decarboxylative condensation of L-alanine and pimeloyl CoA into 7-keto-8-aminopelargonic acid (KAPAS, also known as AON, 8-amino-7-oxononanoate), which is catalyzed by KAPA synthase (EC 2.3.1.47), ~~is~~ the first committed step in the pathway of biotin biosynthesis (Fig. 2), and that was first identified in *E. coli* (Eisenberg and Star, 1968). KAPA synthase, the product of the *bioF* gene from *E. coli*, is a homodimeric and pyridoxal 5'-phosphate (PLP)-dependent enzyme. The molecular mass of the enzyme subunit is about 42 kDa. The enzyme is structurally related to dialkylglycine decarboxylase, a type II aminotransferase when compared to other PLP-dependent enzymes in the amino acid sequence and tertiary structure (Toney et al., 1993). Recent studies by spectroscopic, kinetic, and crystallographic techniques have shown the KAPA synthase from *E. coli* was structurally the apo- and holoform (Alexeev et al., 1998), and the enzyme generates external aldimine complex (Webster et al., 2000). The biotin biosynthesis is understood in detail in microorganisms, but it is relatively poorly understood in plants. Particularly, there is little study on the KAPA synthase of plants. Moreover, of particular interest is the evidence that plants synthesize biotin using the same route as that in *E. coli* (Baldet et al., 1993). Biotin synthesis and utilization in plants have been mainly investigated through analysis of biotinylated proteins (Nikolau et al., 2003; Tissot et al., 1997), and isolation and characterization of auxotrophic mutants (Meinke, 1994). Detailed mutational analysis such as that of auxotrophic mutants has led to an inclusive understanding of biotin synthesis and regulation. The *bio1* auxotroph of *Arabidopsis*, first identified among the collection of recessive embryo-defective mutants, has been shown to be defective in the early step of biotin synthesis, the conversion of KAPA to 7,8-diaminopelargonic acid (DAPA) (Meinke, 1985). The *bio2* mutants have shown to be embryo-defective in the final step of biotin synthesis, the conversion of dethiobiotin to biotin (Patton et al., 1998). These results suggest that the antisense disruption of *AtKAPAS* gene cause lethality in the early stage of plant development. 7-keto-8-aminopelargonate synthase is a pyridoxal 5'-phosphate-dependent 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, microorganisms, and some fungi biosynthesize their own biotin, while animals necessarily require trace amounts of the vitamin in their diet. Therefore, inhibition of the enzymes involving 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 a particular enzyme chosen is indeed essential for a plant growth, and to validate the successful inhibition of the enzyme can lead to an herbicidal effect. Herein, we describe 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*. 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. Individual biotin auxotrophs for KAPA synthase, transformed with anti-sense *Arabidopsis thaliana* KAPAS (*AtKAPAS*) construct, exhibited considerable phenotypic alterations such as growth inhibition, severe growth retardation, yellow–green cotyledons and leaves as well as lethal phenotype (Fig. 3).

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'-phosphate dependent 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 a particular enzyme chosen is indeed essential for a plant growth, and to validate the successful inhibition of the enzyme can lead
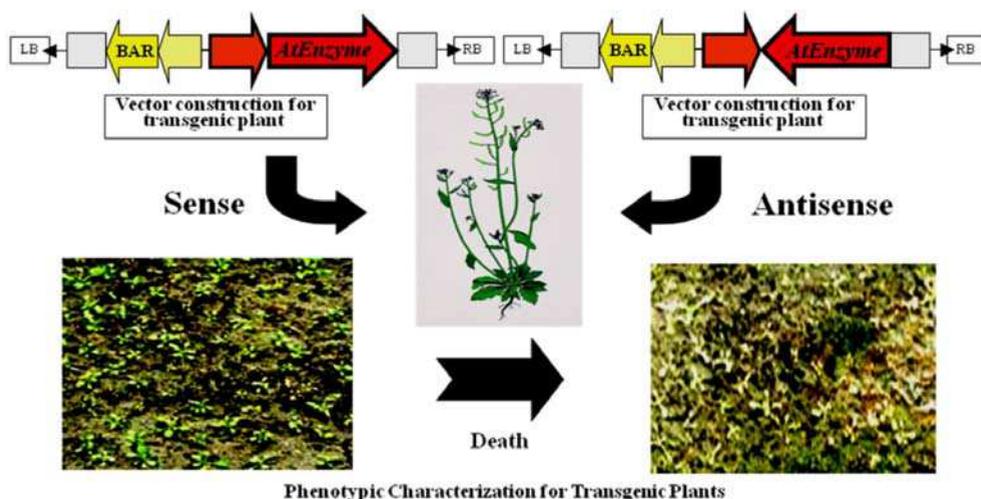

Fig. 3. Sense and Anti-sense Expression of Target Gene in Arabidopsis

to an herbicidal effect. Herein, we describe 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*.

4. Genetic and chemical validation

4.1 *AtKAPAS* from transgenic *E. coli*

Total RNA isolated from leaf tissues of *A. thaliana* was used for preparation of poly(A)+mRNA. 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, the full-length AtKAPAS cDNA was amplified and isolated from *A. thaliana* cDNA library prepared. The primers encompassing the full-length cDNA of *AtKAPAS*, KAPAFB (5'-CAAAAAGAATTCGACGACGACGACAAGATGGCGGATCATTCGTGG GATAAA-3') and KAPARH (5'-GTGCACCTCGAGTTATAATTTGGGAAATAGAAAGGA-3'), were synthesized to include *Eco*RI and *Xho*I restriction site, respectively. Primers of KAPAFB and KAPARH were used in a PCR reaction to amplify the *AtKAPAS*-encoding region. 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). *E. coli* BL21-Gold(DE) (Stratagene, USA) was transformed with expression vector pEMBPek-KAPAS and than cultured in LB (Luria–Bertani broth, USB, USA) medium containing 100 μg·mL^{-1} 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 concentration of 1 mM, and further cultured for 3 h. The culture cells were washed with 50 mM Tris–HCl buffer, pH 8.0, containing 1 mM EDTA, after centrifugation at 9000g for 10 min. The cell pellets were resuspended and pooled in 50 mL of buffer solution (50 mM Tris–HCl, pH 8.0, 200 mM NaCl). The sample was sonicated for 30 s and cooled on ice for 3–5 min, and the procedure was repeated three times. After centrifugation at 1000g for 30 min, the supernatant was purified with MBP affinity chromatography and used as enzyme solution. Eluting fractions separated from *E. coli* transformed with pEMBPek-KAPAS recombinant vector and the control group was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), respectively. SDS–PAGE was performed on a 12% running gel and protein bands were visualized by staining with Coomassie Brilliant Blue G250.

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 may be consisted of *AtKAPAS* protein of 51.3 kDa, and maltose binding peptide MBP affinity tag of 46.9 kDa. For the partial 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 (1.1cm x 30cm, Millipore, USA). The *AtKAPAS* protein binding to MBP resin was eluted with 10 mM maltose solution. To confirm the purification of *AtKAPAS* protein, elutes with *E. coli*-expressed *AtKAPAS* protein and *E. coli* control in the various fractions of affinity chromatography were subjected to SDS-PAGE analysis (Fig. 4). Elutes of *E. coli*-expressed *AtKAPAS* protein contained the induced fusion protein of ca. 98.2 kDa while those of *E. coli* control didn't contain *AtKAPAS* protein.
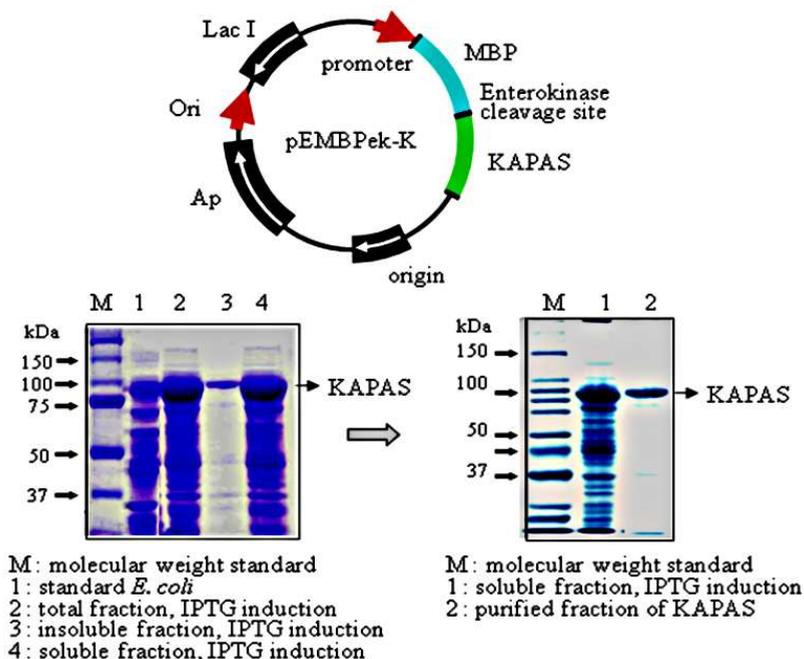
Fig. 4. KAPAS over expression and purification from transgenic *E. coli*.

4.2 *AtKAPAS* inhibition *in vitro* treated with TPTA

For substrate synthesis and enzyme assay *in vitro*, substrate 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) using a linked assay by monitoring the increase in absorption of NADH at 340 nm using a microplate spectrophotometer (Benchmark Plus, Bio-Rad, USA), thermostatically controlled at 37°C. The procedure was the same apart from the reaction volume of 250 μL instead of 1 mL. L-Alanine and pimeloyl-CoA were added to give the desired final concentrations. Prior to analysis, enzyme samples were dialyzed for 2 h at 4°C against 20 mM potassium phosphate (pH 7.5) containing 100 μM pyridoxal 5'-phosphate (PLP). 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.

Enzyme activity was assayed with the partially purified *AtKAPAS* protein extracted from transgenic *E. coli. 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. The dose-dependent *in vitro* inhibition of KAPAS activity by TPTA was noticeably examined and the IC_{50} was calculated as 19.85 μM (Fig. 5).

4.3 Herbicidal activity of TPTA under greenhouse condition

Seeds of *A. thaliana* were sown in plastic pots (24 cm² surface area) filled with artificial nursery soil (Boo-Nong Soil, Seoul, Korea), and the plants were grown to the required
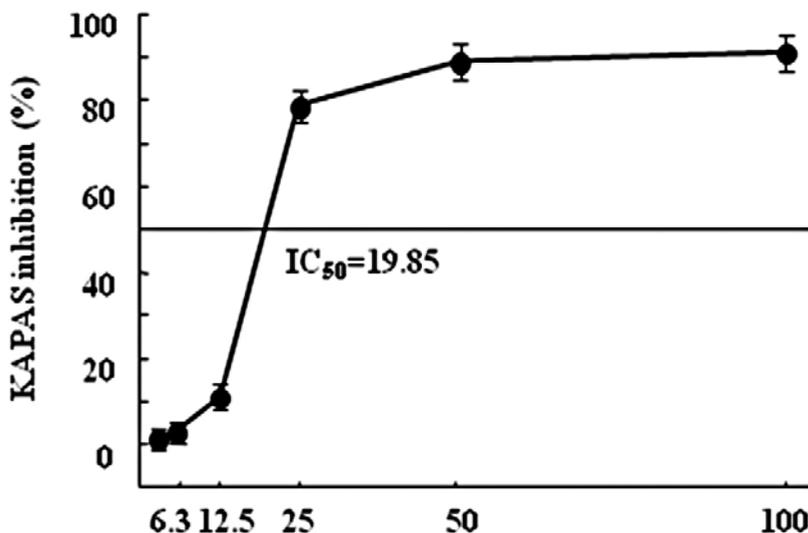
Fig. 5. KAPAS inhibition treated with triphenyltin acetate *in vitro*. Data was expressed as a mean ± S.D.

growth stage for application in a greenhouse maintained at 30~35ºC during the day and 20~25ºC at night. Application was conducted at 40 days after seeding for foliar application of 16, 32, 62.5, 125, 250, and 500 g·ha^{-1} with laboratory spray gun (spray volume of 1000 L·ha^{-1}). The TPTA was used as a solution in acetone/water (60:40 by volume) containing 1.0 g·L^{-1} of Tween-20. The plants were photographed at 1 week after application. The herbicidal spectrum of TPTA was investigated to 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. Foliar application of 0.25, 0.5, 1, 2, and 4 kg·ha^{-1} with laboratory spray gun (spray volume of 1000 L·ha^{-1}) was conducted at 2 weeks after sowing each seeds in plastic pot (350 cm2 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).

The foliar-treatment of 16, 32, 62.5, 125, 250, and 500 g·ha^{-1} TPTA to the 40-day old *A. thaliana* plants has caused herbicidal effects of 8.3, 20, 47, 90, 97, and 100%, respectively. The herbicidal activity was increased as time passed after application. The application rate of more than 125 g·ha^{-1} was shown almost complete death at 1 week after application (Fig. 6). The main symptoms were desiccation and burning effect. Symptoms begun to appear within several hours after application, and the applied region of the leaf was desiccated at 1 day after treatment of more than 250 g·ha^{-1}.

Foliar application of TPTA to 10 weed species was showed good herbicidal activity. The most sensitive species was *Xanthium strumarium* which was completely dead at 250 g·ha^{-1} of TPTA foliar application. *Abutilon avicennae*, *Calystegia japonica*, and *Aeschynomene indica* were also controlled by 500 g·ha^{-1} of TPTA foliar application (Table 1). However, grass weed such as *Sorghum bicolor, Echinochloa crus-galli, Agropyron smithii, Digitaria sanguinalis,* and *Panicum dichotomiflorum* was tolerant to TPTA foliar application comparing to the broad-leaf weeds.
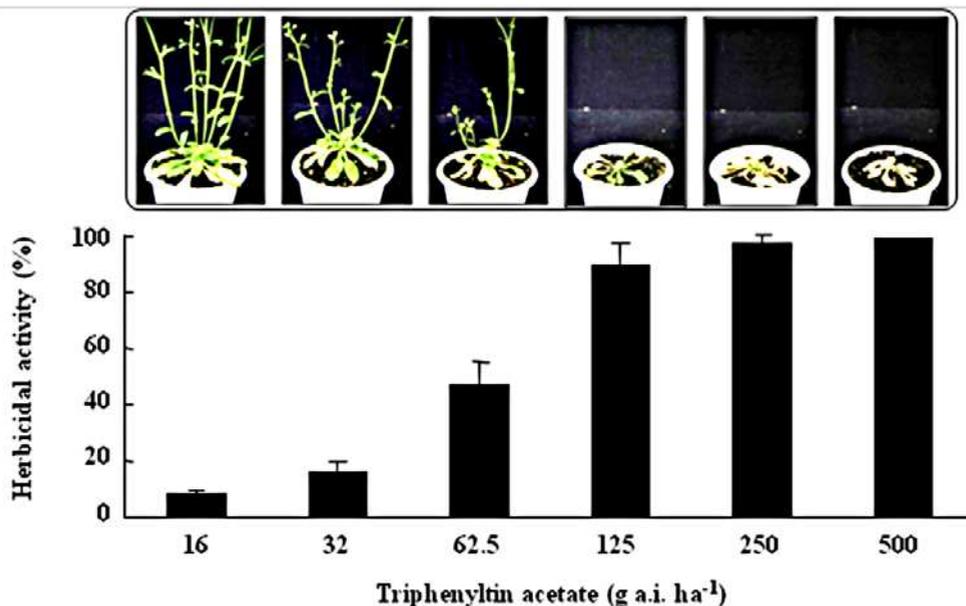
Fig. 6. Herbicidal activity of triphenyltin acetate foliar application at 40 days after seeding under greenhouse condition on the *Arabidopsis thaliana.*

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).

Table 1. Herbicidal activity of triphenyltin acetate on the several weed species under a greenhouse condition

5. Reversal study

Reversal effect was estimated via chlorophyll contents to foliar application and via % germination. 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, 0.063, 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°C, 14/10 h (Light/Dark). The assays were conducted in a completely randomized design with a control and three concentrations of chemicals with three replications. Inhibition percentages at 8 days after treatment were calculated the number of germinated seeds divided by total and the significance level was 0.05 for all analysis.

Plant growth test: *A. thaliana* of 40-day-old plants as reported above were used. Supplement of 1 mM biotin was conducted by foliar laboratory spray gun with spray volume of 5000 L ha^{-1} at each 1 or 2 days before 100 g·ha^{-1} 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). One gram of leaf tissue was placed in a vial containing 7 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and chlorophyll was extracted into the fluid without grinding at room temperature for 24 h in darkness. The extract liquid was transferred to a graduated tube and made up to a total volume of 10 mL with DMSO, and 1.5 mL of the 10 mL aliquots was transferred to microcentrifuge tubes. After centrifuged at 5000g for 10 min, total chlorophyll amount in extracts was determined by the absorbance measurement at 645 nm and 663 nm for each sample using a Microplate Spectrophotometer (Benchmark Plus, Biorad, USA) against DMSO blank. Chlorophyll content was calculated following the equation used by Arnon (1949).

The germination of *A. thaliana* seeds was almost completely inhibited by 0.05 mM TPTA. Also, more than 0.125 mM of TPTA treatment completely inhibited the germination and significantly reduced the plant growth of early stage plants after seed germination. However, the inhibited germination by 0.05 mM TPTA was recovered to 85~92% with the supplement of 0.5 mM biotin, dethiobiotin, and DAPA, except KAPA, one of the biotin biosynthesis intermediates (Fig. 7). Additional supplement of 0.5 mM SAM with 0.5 mM KAPA increased up to 91% of the germination previously inhibited by 0.05 mM TPTA. At 5 days after TPA application, plant leaves were harvested and chlorophyll content was determined following the method reported by Hiscox and Israelstam (1979).

The chlorophyll content in *A. thaliana* plant treated TPTA without biotin pretreatment was 10.7 mg·L^{-1}. The chlorophyll content of the untreated control *A. thaliana* plant was 20.5 mg·L^{-1}, however, the amount of chlorophyll extracted from the *A. thaliana* plant treated with TPTA at 1 and 2 days after biotin pretreatment was 19.5 and 19.8 mg·L^{-1}, respectively. The chlorophyll loss of *A. thaliana* plant treated TPTA was reversed by biotin pretreatment at 1 and 2 days before TPTA application. Consequently, biotin pretreatment reversed the growth inhibition of *A. thaliana* plant treated TPTA at the same extent to the untreated control plants (Fig. 8).
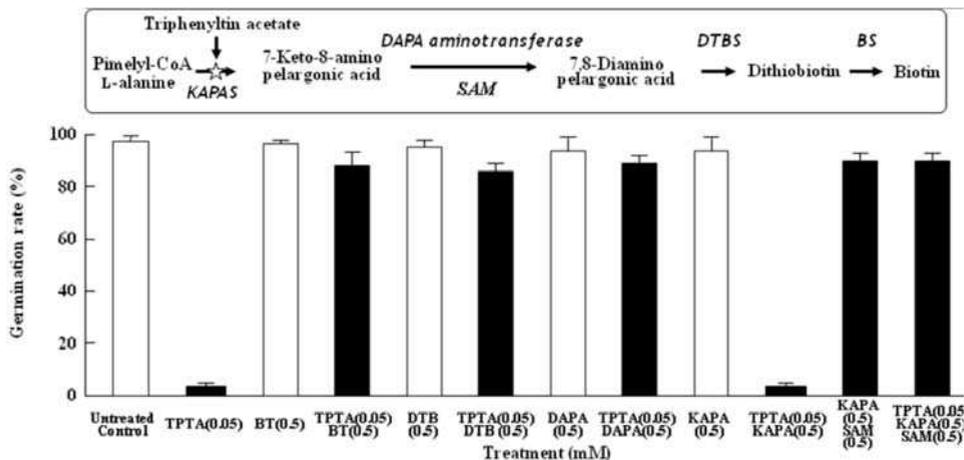
Fig. 7. **Reversal of *A. thaliana* seed germination with biotin biosynthesis intermediates supplement.** KAPAS, 7-keto-8-aminopelargonic acid synthase; DAPA, 7,8-diaminopelargonic acid synthase; DTBS, dithiobiotin synthase; BS, biotin synthase; TPTA, Triphenyltin acetate; BT, Biotin; DTB, dithiobiotin; DAPA, 7,8-diaminopelargonic acid; KAPA, 7-keto-8-aminopelargonic acid; SAM, S-adenosyl-L-methionine.
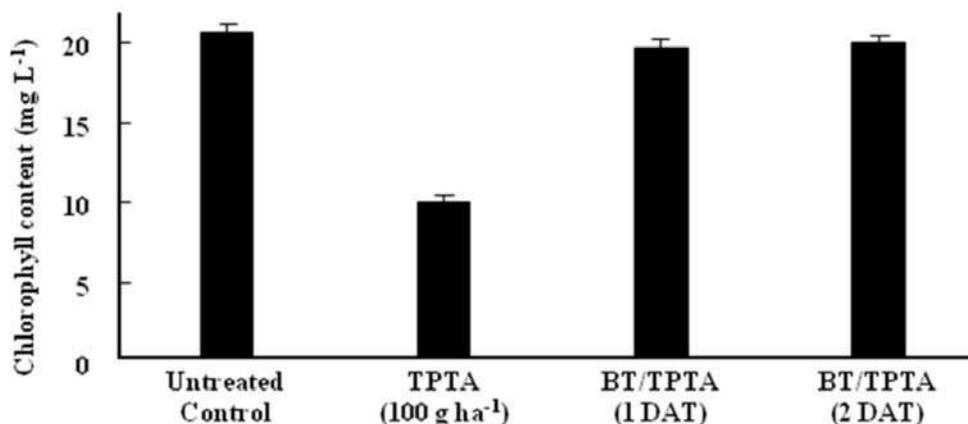

Fig. 8. **Reversal of *A. thaliana* growth inhibition with biotin supplement.** TPTA, Triphenyltin acetate; BT, Biotin; BT/TPTA, BT treatment followed by TPTA; DAT, day after treatment.

5.1 L-alanine accumulation in plants treated with TPTA

Alanine was determined from a detection system of copper complex with L-alanine described by Nakao et al. (1986) with some modification (Lin and Wu, 2005; Weinstein, 1984) from aqueous fraction of extractions. 40-day-old of the Arabidopsis plants grown as reported above was treated with TPTA (200 g·ha⁻¹) by foliar application with laboratory spray gun (spray volume of 1000 L·ha⁻¹). Plant leaves were harvested at 3 days after TPTA

application. Ten grams of plant leaves were homogenized with 100 mL of distilled water and filtered with 2 layers of Mira cloth. The filtrates were separated with equal volume of ethyl acetate. The water fraction was concentrated by vacuum rotary evaporator. The solution (1 mL) was centrifuged at 1000g for 10 min, and chloroform (67 µl) was added to the supernatants, and then centrifuged at 1000g for 10 min, repeatedly. After reaction with 1 mg of NaN_3 and 67 mg of $Cu(OH)_2$ and standing for 20 min at room temperature. 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 $Y = 0.4695X + 0.0146$, $r^2 = 0.9993$.
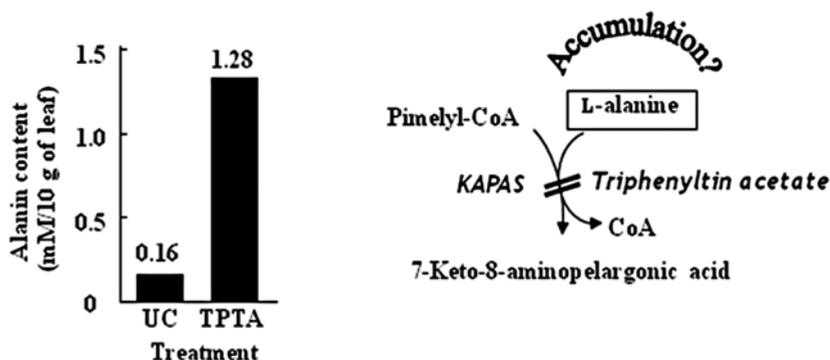
Fig. 9. L-alanin accumulation in *A. thaliana* plants treated with triphenyltin acetate. KAPAS, 7-Keto-8-aminopelargonic acid synthase; UC, untreated control; TPTA, triphenyltin acetate

According to the standard curve, 1.28 mM of L-alanine was detected from *A. thaliana* plants treated with 200 g ·ha^{-1} of TPTA, whereas 0.16 mM of L-alanine from untreated plants. Consequently, the TPTA application induced 8-fold greater L-alanine accumulation in the plants (Fig 9).

5.2 KAPAS gene expression 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 manufacture'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'-GCTGAACGACAAGGAA ATGTTG-3'; KAPAS-R, 5'-GAGTGGCTGTGTTGTCAAAG-3'. Primers for amplification of reference gene, tubulin was: TUB-F, 5'-CTCAAGAGGTTCTCAGCAGTA-3'; TUB-R, 5'-TCACCTTCTTCATCCGCAGTT-3'.

To expand our understanding on the role of TPTA, the expression of KAPAS gene 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^{-1} TPTA (Fig. 8). KAPAS was expressed in most tissues, with the highest levels either in stems or roots of the untreated plants, and tubulin was also showed good reference gene expression in *A. thaliana*. However, RNA expression of KAPAS band was indeed fainter or disappeared in the lane representing leaf tissue of TPTA (+) plants. Also, slightly less RNA appeared to the tubulin band than in the other lane. This result implying that the TPTA treatment is severely subjected to protein KAPAS translation and/or post translational regulation in the leaf within 1 day of treatment like as *bio 1* mutants.
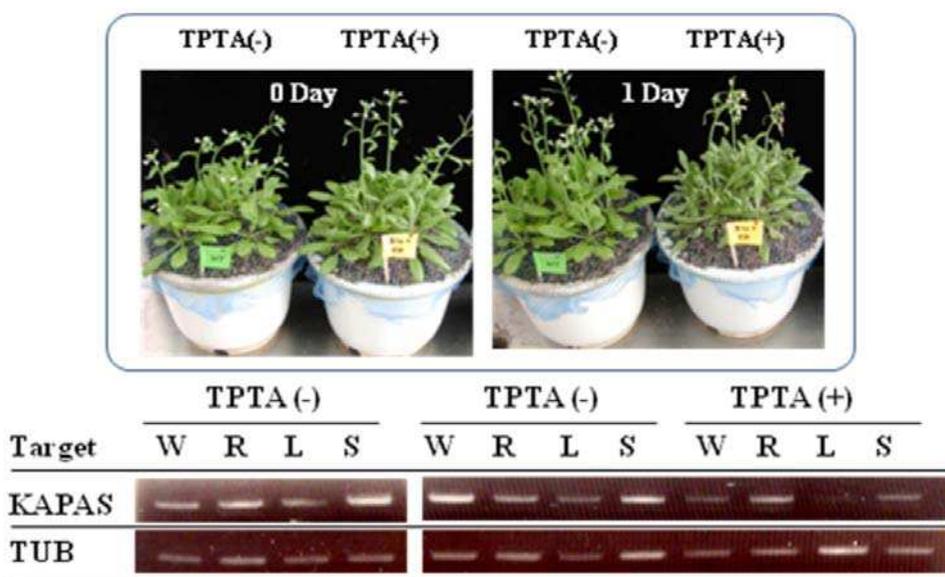

Fig. 8. Semi-quantitative RT-PCR analysis of KAPAS gene expression in *A. thaliana* plants. TPTA(+/-), treatment with/without 100 g ha^{-1} triphenyltin acetate; W, whole plant; R, root; L, leaf; S, stem; KAPAS, 7-Keto-8-aminopelargonic acid synthase; TUB, tubulin; Analysis was conducted 1 day after TPTA treatment.

6. Summary

As a number of enzymes related in the metabolic pathways of plants are essential for the growth and development, those can be utilized as potential herbicide targets. We have performed molecular genetic dissection using reverse genetics of antisense 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 (Hwang et al., 2003; 2010).

Many researchers have investigated the KAPAS in microorganisms and the most of these reports were focused on the biosynthesis in microorganisms (Eisenberg and Star, 1968),

purification and characterization (Ploux 1992, Stoner and Eisenberg, 1975a; 1975b), 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). 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 microorganisms, and thus, inhibition of the enzymes involved in the pathway is potential and attractive target for both herbicide and antibiotics development. These evidences strongly support the hypothesis that inhibitors of microbial enzymes of the biotin biosynthesis pathway might exhibit herbicidal properties as a biofunctional inhibitor.

For instance, two natural compounds isolated form culture filtrates of *Streptomyces* species, actithiazic acid and amiclenomycin, are biotin synthase, final step of biotin biosynthesis pathway, inhibitors of mycobacteria and plants. Ashkenazi et al (2005, 2007) reported the analogs of KAPA and DAPA, possessing chain lengths of eight carbon atoms, 7-aminooctanoic acid hydrochloride, 7-allyloxy-6-oxo-octanoic acid, and 6,7-diaminooctanoic acid dihydrochloride displayed a inhibitors of biotin biosynthesis as potential herbicides. In biotin biosynthesis pathway, four steps of enzymes, KAPAS, DAPA amino transferase, DTBS, and biotin synthase, were working continuously. Among them, the first step of KAPAS inhibitors was not introduced until now.

From these backgrounds, we studied KAPAS inhibition using various commercialized compounds as pesticides. Among them, the chemical TPTA was selected *in vitro* assay with *AtKAPAS* over-expressed from transgenic *E. coli*. Also, we investigated a genetic and chemical validation of the compound as a potential lead compound for KAPAS inhibitors under greenhouse condition *in vivo*. KAPAS activity was completely inhibited by 100 μM of TPTA *in vitro* enzyme assay with the IC_{50} value of 19.85 μM. The germination of *A. thaliana* seeds was also completely inhibited when TPTA concentration was greater than 63 μM. The foliar-treatment of more than 125 g·ha^{-1} TPTA to the 40-day old *A. thaliana* plants has caused almost complete death. Also, foliar application of TPTA to the 10 weed species was showed good herbicidal activity under a greenhouse condition.

Abell (1996) and Pillmoor (1995) suggested that if a protein is a potential target, a 60~80% inhibition of its activity leads to a severe growth phenotype. In accordance with this standpoint, our results suggest that the KAPAS might be a good target enzyme for new herbicide development. However, these results were not sufficient to explain the exact mechanism of action of TPTA as one of the KAPAS inhibitors. It is important to emphasize that the correlation between *in vitro* and *in vivo* inhibition patterns could be measured reproducibly and confirmed with reversal effect with the supplement of biotin and intermediates in the biotin biosynthesis pathway and/or substrate accumulation and/or RNA expression pattern in plants.

The supplement of biotin or biotin biosynthesis intermediates, except KAPA, was induced the germination and growth rescue previously inhibited by TPTA. The KAPA, even one of the biotin biosynthetic precursors, supplement could not rescue the germination inhibited by the compound TPTA, but additional supplement of 0.5 mM SAM increased up to 91% of the germination inhibited by 0.05 mM TPTA. In the same way, the antisense auxotrophs were rescued by supplementing of biotin (Hwang et al., 2003; 2010). From these results, we

firstly reported the SAM is essential donor of amino group for synthesis of the biotin precursor DAPA in plants. DAPA aminotransferase is a pyridoxal 5'-phosphate (PLP) enzyme that catalyzes the transamination of KAPA to yield DAPA (Eisenberg and Stoner, 1971, Stoner and Eisenberg, 1975). In *E. coli*, the amino donor in this reaction is SAM (Breen et al., 2003). The enzyme from *E. coli* has been well characterized and its 3D structure determined.

Detailed mutational analysis of auxotrophic mutants has led to an inclusive understanding of biotin synthesis and regulation. The *bio1* auxotroph of *Arabidopsis*, first identified among the collection of recessive embryo-defective mutants, was shown to be defective in the early step of biotin synthesis, the conversion of KAPA to DAPA (Breen et al., 2003). Mutant *bio1*, first plant auxotroph for biotin, has shown to result in embryonic lethality, and its embryos remain pale throughout development, typically arrested between germination and cotyledon stage of embryogenesis (Alban et al., 2000, Meinke, 1985). Plant growth was rescued by biotin, dethiobiotin, or DAPA, but KAPA supply, or by genetic complementation by *E. coli bioA* gene coding DAPA aminotransferase, demonstrating that mutant plants are defective in this enzyme (Alban et al., 2000; Meinke, 1985; Shellhammer and Meinke, 1990; Patton et al., 1996; 1998). Based on feeding studies, Shellhammer and Meinke (1990) suggested that *bio 1* was defective in the conversion of KAPA to DAPA, the enzymatic function of the *BioA* protein of *E. coli*. This is the reason of the conversion of KAPA to DAPA in plant needs SAM supplement for rescue in mutant *bio 1* after treatment with TPTA, and appears to follow the same pattern as identified for *E. coli* (Shellhammer and Meinke, 1990; Patton et al., 1996; 1998). With these results, we firstly reported the SAM is an essential donor of amino group for the conversion of KAPA to DAPA in plants (Fig. 9).
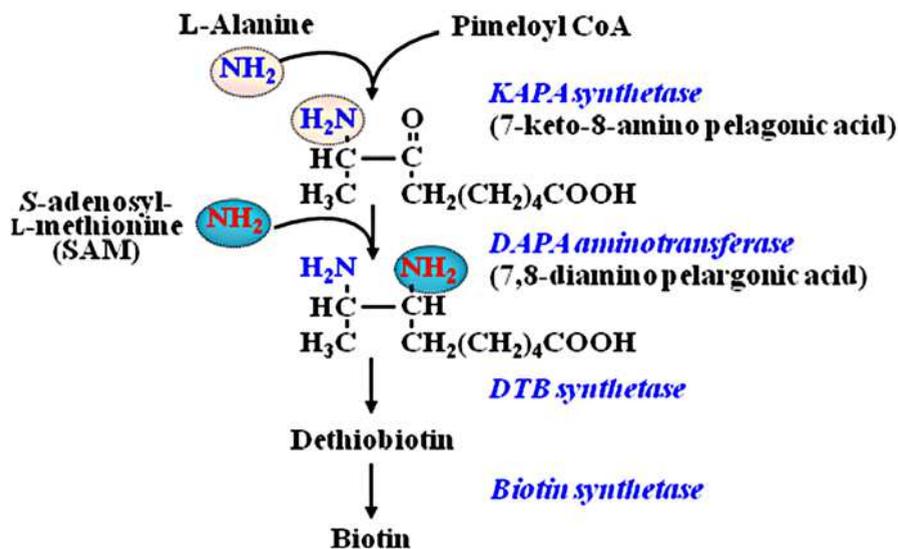

Fig. 9. Suggestion of S-adenoxyl-L-methione (SAM) is essential donor of amino group for the conversion of KAPA to DAPA in plants.

Furthermore, TPTA induced 8-fold greater accumulation of L-alanine, a substrate of KAPAS, in the foliar-treated plants. Also, RNA expression band for KAPAS was

disappeared or indeed fainter in the lane representing leaf tissue treated with TPTA. This result suggested that the TPTA treatment is subjected to protein KAPAS translation and/or post translational regulation in the leaf like as *bio 1* mutants within 1 day of treatment. Also, TPTA showed slight inhibition to the tubulin translation and/or post translational regulation. Kourai et al. (1973) reported the mode of action of TPTA against *E. coli*. TPTA was only slightly inhibited respiration, permeability, protein synthesis and cell wall synthesis, but markedly inhibited RNA and DNA synthesis by *E. coli.* The antimicrobial action of TPTA was reversed by cysteine and 2-mercaptoethanol, and the active site of this compound is the metal atoms.

These results show that the action of TPTA was co-related with the enzyme activity of KAPAS in plants and RNA synthesis, coincidently. Because, TPTA inhibited the activity of KAPAS *in vitro*, germination of *A. thaliana* seeds *in vivo*, and the growth of weeds in a greenhouse condition. Also, TPTA inhibited the RNA expression in the leaf tissue of *A. thaliana*. This inhibition of seed germination was rescued by coincident treatment of KAPA and SAM, but could not rescue by supplement of KAPA only. It is not sure that the metal atoms of TPTA act on the active site or a cofactor was needed, but these results suggested that the KAPAS is a potential herbicidal target site in the biotin biosynthesis pathway, and TPTA is one of the KAPAS inhibiting chemicals even if the compound have been used as one of the fungicides.

 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 (Dakshinamurti and Bhagavan, 1985; Jelenska et al., 2002; Pinon et al., 2005; Nikolau et al., 2003).

These biotin-dependent carboxylases in plants include cytosolic acetyl-CoA carboxylase, chloroplastic geranyl-CoA and acetyl-CoA carboxylases, and mitochondrial methylcrotonoyl-CoA carboxylase (Alban et al., 2000; Nikolau et al., 2003). 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).

The reducing level of this enzyme activity required as a commercial herbicide target is hard to assume at present time. However, it appears that complete inhibition of enzyme activity at these targets is not necessary for plant death (Abell, 1996). In mutant plants with reduced amounts of glutamine synthetase activity, the target of glufosinate, reduction in glutamine synthetase activity of only 38% was sufficient to cause severe abnormalities (Blackwell et al., 1987). Antisense knock-out of acetolactate synthase (ALS), the target site of the sulfonylureas, imidazolinones, and triazolopyrimidines can produce plants displaying a range of ALS inhibitor-like symptoms such as growth retardation and necrosis (Blackwell et al., 1987; Höfgen, 1995). Such directed knock-outs allow the screening of enzymes whose inhibition might be expected to have catastrophic effects in the plant, based on knowledge of pathway dynamics. However, our knowledge of biochemical pathways in plants is incomplete and the next major herbicide target may lie in an unexpected area of plant

metabolism. Generally, it can be argued that we still do not know in detail how plants actually die as a result of inhibition of some known targets.

Even though whole plant screening will remain central to agrochemical discovery, high-throughput biochemical screening might be effective to accelerate the discovery of novel compounds. For example, these can allow the detection of hits that may be missed in glasshouse screens due to poor plant bioavailability, or the rapid and thorough evaluation of a target site by concerted screening against diverse sets of chemistry. Structure-activity relationships can provide inspiration for further chemical synthesis based on binding hypotheses or single parameter data not available from glasshouse screening. Further rationalization of activities and downstream of genomics such as high-throughput x-ray crystallography for three-dimensional analysis of protein-inhibitor interactions (structural genomics) will assist in developing 'virtual' or '*in silico*' screening of chemistry. Greater reliance on high-throughput biochemical screening will necessitate an improved ability to convert *in vitro* hits into biologically active molecules through a better understanding of whole plant-compound interactions and improved test systems would be confirmative for this speculation.

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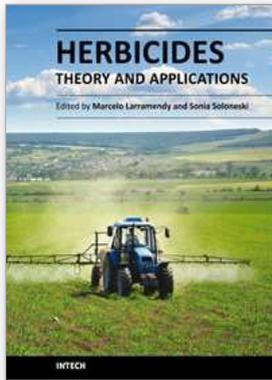
Herbicides, Theory and Applications

Edited by Prof. Marcelo Larramendy

The content selected in Herbicides, Theory and Applications is intended to provide researchers, producers and consumers of herbicides an overview of the latest scientific achievements. Although we are dealing with many diverse and different topics, we have tried to compile this "raw material" into three major sections in search of clarity and order - Weed Control and Crop Management, Analytical Techniques of Herbicide Detection and Herbicide Toxicity and Further Applications. The editors hope that this book will continue to meet the expectations and needs of all interested in the methodology of use of herbicides, weed control as well as problems related to its use, abuse and misuse.

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