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NOVEL TECHNIQUES

A Bacterial Indole-3-acetyl-L-aspartic Acid Hydrolase Inhibits Mung Bean (*Vigna radiata* L.) Seed Germination Through Arginine-rich Intracellular Delivery

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Abstract Indole-3-acetyl-L-aspartic acid (IAA-Asp) is a natural product in many plant species and plays many important roles in auxin metabolism and plant physiology. IAA-Asp hydrolysis activity is, therefore, believed to affect plant physiology through changes in IAA metabolism in plants. We applied a newly discovered technique, arginine-rich intracellular delivery (AID), to deliver a bacterial IAA-Asp hydrolase into cells of mung bean (*Vigna radiata*) seeds and measured its effects on mung bean seed germination. IAA-Asp hydrolase inhibited seed germination about 12 h after the enzyme was delivered into cells of mung bean seeds both covalently and noncovalently. Mung bean seed germination was delayed by 36 h when the enzyme protein was noncovalently attached to the AID peptide and longer than 60 h when the enzyme protein was covalently attached to the AID peptide. Root elongation of mung bean plants was inhibited as much as 90% or 80%, respectively, when the IAA-Asp hydrolase was delivered with the AID peptide by covalent or noncovalent association. Further thin-layer chromatography analysis of plant extracts indicated that the levels of IAA increased about 12 h after treatment and reached their peak at 24 h. This result suggests that IAA-Asp hydrolase may increase IAA levels and inhibit seed germination of mung bean plants and that the AID peptide is a new, rapid, and efficient experimental tool to study the *in vivo* activity of enzymes of interest in plant cells.

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Introduction

Indole-3-acetyl-L-aspartic acid (IAA-Asp) is a natural product in many plant species and plays many important roles in auxin metabolism and plant physiology. First, IAA-Asp levels can be dramatically increased in plants when applied with high doses of exogenous auxins (Andreae and Good 1955). Second, it is the last intermediate retaining the intact indole ring in the IAA nondecarboxylative oxidation pathway in most plants (Tuominen and others 1994; Normanly 1997; Ostin and others 1998). Finally, in henbane cell cultures, IAA-Asp is the main IAA conjugate accumulated in a high-temperature-resistant line whereas IAA-glucose is the main IAA conjugate in a nonresistant variety (Oetiker and Aeschbacher 1997). Therefore, IAA-Asp may function in plants as a novel product of IAA degradation and antistress processes.

Because IAA-Asp is the last intermediate retaining the intact indole ring in the IAA nondecarboxylative oxidation pathway in most plants, an enzyme capable of hydrolyzing IAA-Asp would be of great interest. Such an enzyme has not been found yet in plants. It is therefore difficult to further investigate IAA-Asp function in plants because of the lack of genetic tools. The first enzyme (and its gene) capable of specifically hydrolyzing IAA-Asp was found in our earlier study of the bacterium *Enterobacter agglomerans* (Chou and others 1996, 1998). Using a transgenic approach and this bacterial IAA-Asp hydrolase (IAASPH) gene in *Arabidopsis thaliana*, IAASPH was found to significantly decrease IAA-Asp levels (Tam and Normanly

2002). The transgenic study demonstrated that the bacterial enzyme can also function in plants and, therefore, provided a potential tool for the study of IAA-Asp function in plants.

Even though the transgenic approach has shown significant activity of IAA-Asp hydrolase in plants, a noticeable phenotype is still lacking. In our recent studies (Chang and others 2005; Wang and others 2006), the so-called "arginine-rich intracellular delivery (AID)" peptides were found capable of delivering foreign proteins such as green fluorescence protein (GFP) and red fluorescence protein (RFP) into plant cells without causing damage to the cells. This transmembrane activity was achieved by proteins containing protein transduction domain (PTD) sequences (Schwarze and others 1999). The three most studied PTD sequences are found in the human immunodeficiency virus type-1 (HIV-1) transcriptional activator Tat protein (Frankel and Pabo 1988; Green and Loewenstein 1988), the *Drosophila* homeodomain transcription factor antennapedia (Joliot and others 1991), and the herpes simplex virus structural protein VP22 (Elliott and O'Hare 1997). The main components of PTDs are positively charged, basic amino acids. Cellular uptake of polyarginine tends to be more efficient than that of polylysine, polyhistidine, or polyornithine (Futaki 2002). Various chain lengths of polyarginine were examined for cellular internalization and the highest translocation efficiency was reached by using octa-arginine or nona-arginine peptides (Futaki 2002). The PTD sequence delivering process therefore is called "arginine-rich intracellular delivery" and the peptides containing such PTD are called AID peptides.

In our previous studies we demonstrated that PTDs can deliver proteins across cellular membranes in covalent or noncovalent fashion in plants (Chang and others 2005; Wang and others 2006). These results provide plant biologists with a much easier way to investigate enzyme or protein activities in plants *in vivo* rather than the time-consuming transgenic approach. Here, we present the first report using the AID peptide to assay the activity of IAA-Asp hydrolase in seed germination of *Vigna radiata*, a recalcitrant grain legume for *Agrobacterium*-mediated gene transformation (Jaiwal and others 2001).

Materials and Methods

Plasmid Constructions

Schematic constructs of plasmids used in protein transduction are shown in Figure 1. The pIAASPH plasmid was generated based on the pETBlue-1 expression vector (Novagen, Madison, WI, USA) containing the IAASPH gene (Chou and others 1998) and in-frame inserted with six histidine residue sequence. The pIAASPH-Tat plasmid is derived from pIAASPH with an in-frame insertion of Tat AID peptide sequence. The pIAASPH-Tat plasmid was generated by digestion of pIAASPH with the *Eco*R1 restriction enzyme and followed by insertion of the DNA sequence encoding 11 amino acids (NH_2-YGRKKRRQRR R-COOH) that was generated by annealing of the primers Tat-up (5′-AATTTACGGCC GGCCGCAAGAAACGCC GCCAGCGCCGCCGC-3′) and Tat-down (5′-AATTGCG GCGGCGCTGGCGGCGTTTCTTGCGGCCGTA-3′). The pTat-HA plasmid (kindly provided by Dr. Steven F. Dowdy, Washington University, St Louis, MO, USA) consists of 11 amino acids of HIV-Tat PTD (Tat) in-frame with six histidine residues and a hemagglutinin (HA) tag under the control of the T7 promoter.

Protein Preparation

The three plasmids (pIAASPH, pIAASPH-Tat, pTat-HA) were heat-shocked transformed into the *E. coli* Turner (DE3) p*lac*I strain (Novagen) and then cultured aerobically at 37°C in 500 ml of LB broth (1% casein digest peptone, 0.5% yeast extract, and 200 mM NaCl, pH 7.5) containing 100 μg ml^{-1} of ampicillin and chloramphenical. The isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM when the OD_{600} of bacterial culture reached 0.8 for induction of protein expression. After 4 h of induction with IPTG, cells were harvested by centrifugation and the pellets were resuspended in 4 ml of 50 mM phosphate buffer at pH 8. Cells were then disrupted by sonication (Microson XL, Misonix, NY, USA), and the insoluble debris was removed by centrifugation at 4°C. The collected supernatants were applied directly onto a His-Bind column (Bio-Rad, Hercules, CA, USA) containing chelating Sepharose fast flow resin (Amersham Biosciences, Piscataway, NJ, USA) for protein purification based on the manufacturer's manual. Protein

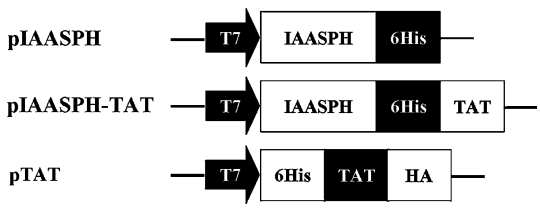

Fig. 1 Schematic structures of plasmids used in protein transduction. pIAASPH is the IAASPH gene in the pETBlue1 expression vector and contains an in-frame C-terminal His_6-tagged (6His) sequence under the control of a T7 promoter. pIAASPH-TAT is the pIAASPH in-frame inserted with a Tat protein AID peptide (YGRKKRRQRRR). pTat-HA consists of the 11-amino-acid sequence of HIV-Tat PTD in-frame with 6His tag and a hemagglutinin (HA) rag under the control of a T7 promoter

concentrations were determined using a Coomassie dye-based protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) according to the method of Bradford (1976). Protein purity was analyzed by SDS-PAGE based on the method of Laemmli (1970).

Enzyme Activity Assays

IAASPH activity was analyzed as described by Chou and others (1996). In short, 100 µl of 50 mM phosphate buffer containing 10 µg of protein and 1 mM IAA-Asp was incubated at 30°C for 10 min. The assay was then terminated by 20 µl of 85% phosphoric acid after 10 min of reaction. Two hundred microliters of water-saturated ethyl acetate was added to the reaction mixture to extract the nonpolar compounds, including IAA and IAA-Asp. The ethyl acetate extracts were analyzed by silica gel 60-F_{254} thin-layer chromatography (TLC) (Merck, Darmstadt, Germany) using a solvent system of chloroform:methanol:H_2O (85:14:1 v/v/v). After air-drying at room temperature, the TLC plate was developed by immersion in Ehmann's reagent (Ehmann 1977) for about 5 s and was incubated at 100°C for 1 min. Both IAA and IAA-Asp could be visualized by their bright blue color after development. Under these conditions, IAA had an R_f value of 0.5 and IAA-Asp had an R_f value of 0.1.

Protein Internalization

Covalent Protein Transduction Mung bean seeds were immersed in 2 ml of 10 mM phosphate buffer for 4 h and washed with water as a negative control at 25°C. To test

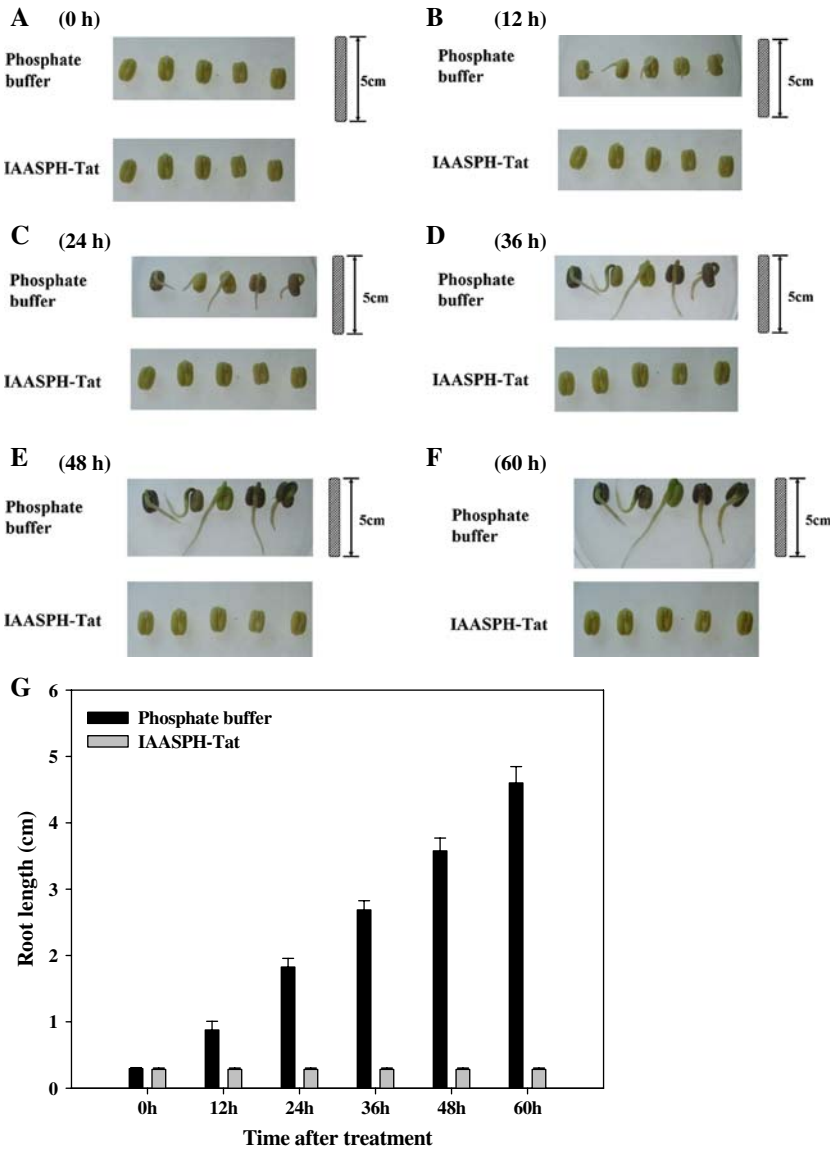

Fig. 2 Effects of IAASPH on mung bean seed germination through AID peptide activity covalently

covalent protein transduction, mung bean seeds were immersed in 2 ml of 10 mM phosphate buffer containing 400 μg of IAASPH-Tat for 4 h and washed with water. The treated seeds were placed in Petri dishes at 25°C for observation of seed germination. After recording seed germination, the seedlings were collected and stored at −80°C for further TLC analysis.

Noncovalent Protein Transduction As above, mung bean seeds were immersed in 2 ml of 10 mM phosphate buffer for 4 h and washed with water as a negative control at 25°C. In addition, 400 μg of IAASPH or Tat alone was added in the treatment as another control for noncovalent protein transduction assays. To test noncovalent protein transduction, 1 ml of 400-μg IAASPH was mixed with 1 ml of 400-μg Tat and washed with water. The treated seeds were placed in Petri dishes at 25°C for observation of seed germination. After recording seed germination, the seedlings were collected and stored at −80°C for further TLC analysis.

Plant Extraction for TLC Analysis

The −80°C frozen mung beans were ground to a fine powder with liquid nitrogen and extracted with 2 ml of cold extraction buffer (ethanol:20 mM KH_2PO_4 = 1:9 v/v). The extracts were centrifuged at 13,000 rpm for 20 min. Supernatants were filtered through 0.20-μm filters (MFS-13,

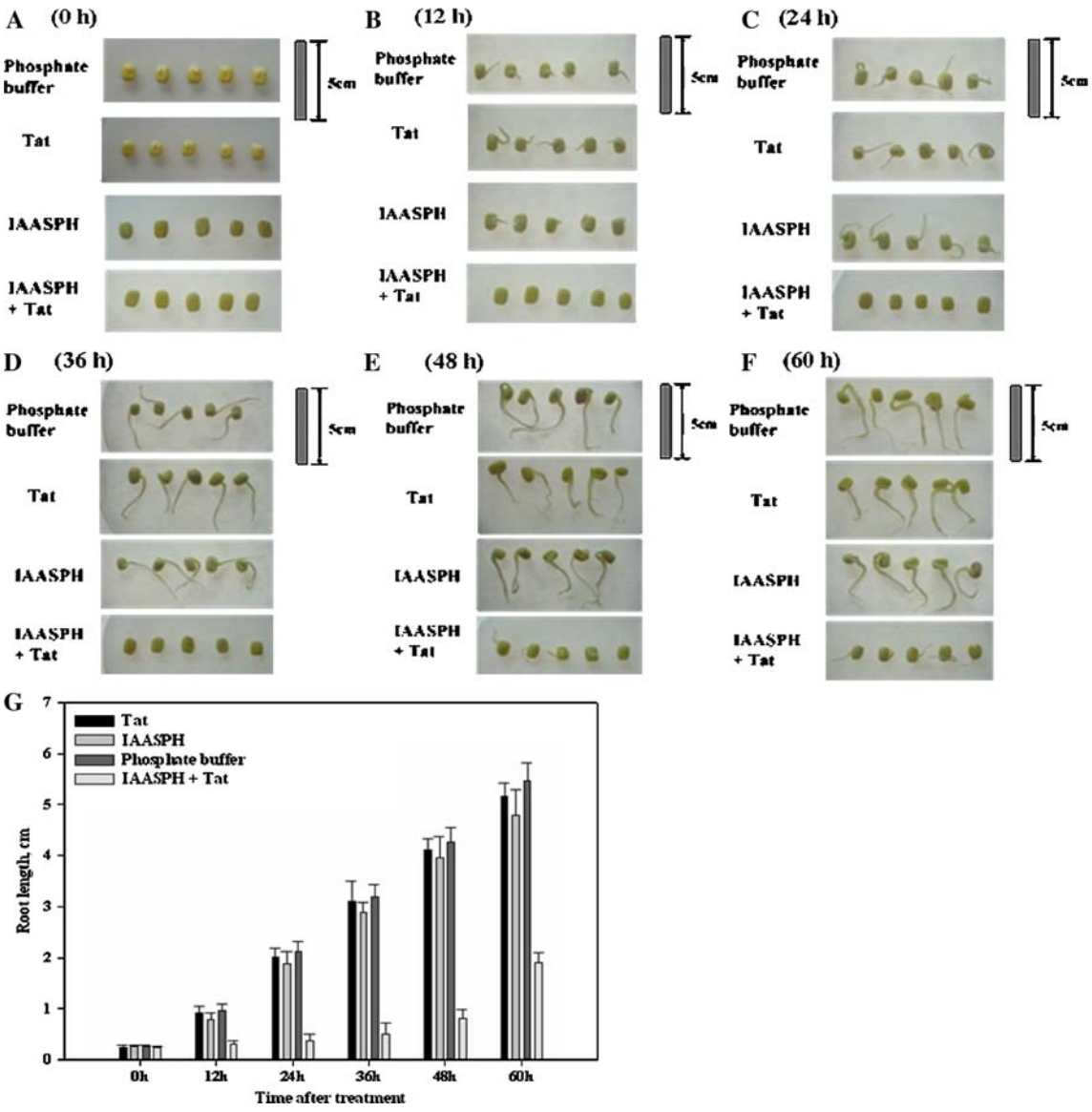

Fig. 3 Effects of IAASPH on mung bean seed germination through AID peptide activity noncovalently

Advantec MFS) with a plastic disposable injector and kept under −20°C until analysis. The filtrates were then subjected to TLC analysis as described above.

Results and Discussion

Enzyme Activities Through Protein Transduction

There are still debates about the mechanism of arginine-rich intracellular delivery for protein internalization. Recent reports proposed that an electrostatic interaction first takes place between cationic AID peptides and the negatively charged polar heads of the phospholipids of the plasma membrane. Subsequently, a specialized form of endocytosis, called macropinocytosis, takes place in the uptake of AID peptides and its cargoes (Snyder and Dowdy 2004; Nakase and others 2004; Kaplan and others 2005). In a separate study, we found that the plant AID processes could be inhibited by a macropinocytosis inhibitor and this supported the hypothesis that the protein internalization mechanism in plants also includes macropinocytosis (Chang and other 2007). However, more detailed studies of the molecular mechanism are needed.

In the covalent protein transduction experiments, the IAASPH-Tat fusion protein strongly inhibited mung bean seed germination when mixed with mung bean seeds in phosphate buffer for 4 h (Figure 2). In the control experiment containing only 10 mM phosphate buffer at pH 8, seeds started to germinate after 12 h of incubation at 25°C (Figure 2B). However, seeds in the IAASPH-Tat treatment experiment did not germinate until 72 h of incubation (data not shown). After 60 h of incubation, the average root length of control plants was 4.6 cm, but in treatment plants

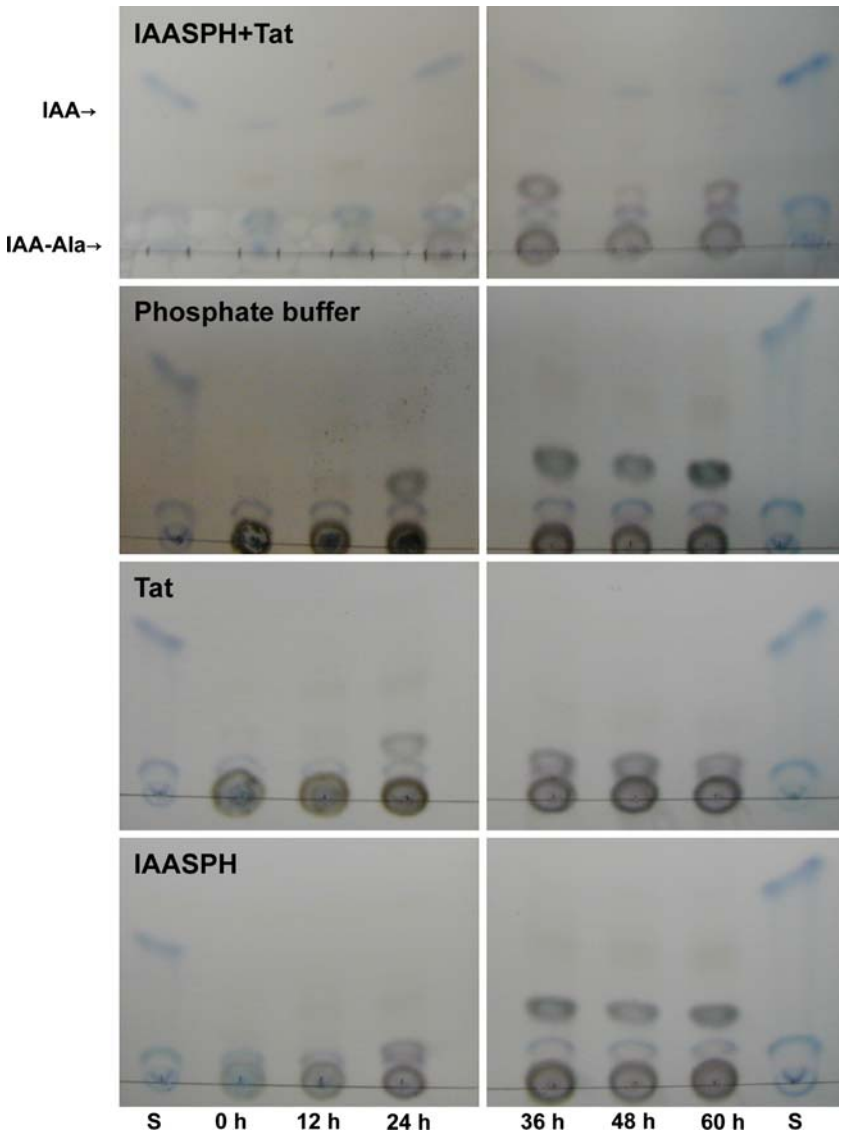

Fig. 4 The thin-layer chromatography (TLC) analysis of mung bean extracts. The TLC plates were developed by Ehmann's reagent for detection of indole compounds. The "S" was a standard mixture of 20 ng IAA and 20 ng IAA-Ala on the left and 50 ng IAA and 50 ng IAA-Ala on the right

it remained at 0.2 cm (Figure 2G), strongly suggesting that the activity of IAASPH-Tat inhibited mung bean seed germination.

In the noncovalent protein transduction experiments, IAASPH was mixed with Tat AID peptide noncovalently for the treatment experiment, and three other experiments were designed as controls: phosphate buffer only, Tat in phosphate buffer, and IAASPH in phosphate buffer. The results showed that only IAASPH mixed with Tat AID peptide (IAASPH + Tat) inhibited mung bean seed germination (Figure 3). Similar to the covalent protein transduction study, control plants started to germinate after 12 h of incubation, and the "IAASPH + Tat" treatment started to germinate after 36 h of incubation (Figure 3G). The growth rate of root elongation, however, was slower for "IAASPH + Tat" compared to the control plants. These results indicate that the efficiency of covalent protein transduction is better than that of noncovalent protein transduction. Nevertheless, both experiments showed a strong inhibitory effect of IAASPH on mung bean seed germination.

TLC Analysis of Plant Extracts

After the measurements of root length of each experimental plant, the plant samples were stored at −80°C for further TLC analysis. To analyze the major indole compound profiles, frozen mung bean plants were ground to powder with liquid nitrogen and extracted with 2 ml of cold extraction buffer based on Sen and others (1997). In TLC analysis, a blue spot corresponding to the IAA spot position was found in each sample of "IAASPH + Tat" time course experiment, with its peak at 24 h (Figure 4). No blue spot was found in any other control experiment. The "smile" shapes of the blue spots probably were caused by the interaction between the nonpolar TLC solvent system and the trace of polar extraction buffer. However, a clear trend could be drawn from the TLC plates. The increase of IAA spots and the inhibition of mung bean seed germination suggest that IAASPH in plant cells after AID peptide cellular internalization caused the inhibition of mung bean seed germination. After 24 h of incubation, the IAASPH might be degraded by proteases of plant cells and the increased IAA levels were redirected to other catabolic pathways or storage forms.

There are two possible sources of IAA-Asp for IAASPH activity. First, the mung bean plants may use IAA-Asp to store IAA in seed. This reserve was significantly hydrolyzed by AID-delivered IAASPH and, therefore, quickly increased the IAA level and inhibited seed germination. Second, in mung bean plants the IAA nondecarboxylative oxidation pathway (Tuominen and others 1994; Normanly 1997) may be the major IAA degradation pathway. Because IAA-Asp is a key intermediate of the IAA nondecarboxylative oxidation pathway in most plants, IAASPH may be able to indirectly block the processes of the IAA nondecarboxylative oxidation pathway by hydrolyzing IAA-Asp back to IAA. Further analysis of mung bean IAA catabolism is needed to clarify this issue.

In conclusion, we have presented a new, novel, and simple technique for plant biologists to assay proteins or enzymes *in planta* without using the time-consuming transgenic approach. The AID peptides are inexpensive, nontoxic, fast, and easy materials for most plant biology laboratories to prepare and handle. It may open a new way for experimental designs in plant research.

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