Characterization of Arabidopsis Histidine Kinase 3 and Proteomic Analysis of Its Mutant

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Histidine kinase plays important roles in signal transduction in plant. We characterized the function of Arabidopsis histidine kinase 3 (AHK3) and analyzed the expression patterns of genes and proteins in its mutant *ahk3* by *trans*-zeatin (*t*-zeatin). The *ahk3* exhibited decreased sensitivity to *t*-zeatin during callus formation, seedling growth, and leaf senescence. From proteomic analysis of *ahk3*, eukaryotic translation initiation factor 5A-2, auxin binding glutathione *S*-transferase, and NDPK1 were identified not to be induced by *t*-zeatin, when compared to the wild-type. In addition, the expression levels of *ARR4* and *ARR16* among A-type response regulators (ARRs) markedly decreased in *ahk3* by *t*-zeatin treatment. These results suggest that AHK3 plays an important role in cytokinin signaling and the proteins identified from proteomic analysis and specific ARRs, ARR4 and ARR16 may be directly or indirectly associated in AHK3-mediated cytokinin signaling.

Key words – Arabidopsis histidine kinase 3, proteomics, response regulators, *t*-zeatin

Introduction

Cytokinins have been recognized as essential plant hormones involved in a wide range of plant growth and physiological processes such as cell division, shoot initiation, leaf and root differentiation, chloroplast biogenesis, leaf senescence, and stress response[16]. Recent studies suggest that His-to-Asp phosphorelay mechanism (also known as two-component system) is involved in various plant signal transductions, including cytokinin signaling[9,23]. This system consists of a histidine kinase that senses a signal input and a response regulator that mediates the output[23]. Up to date, at least sixteen putative histidine kinases (AHKs) and ten members of A-type response regulators (ARRs) were identified in Arabidopsis[4,9].

Four AHKs have been known to be involved in cytokinin signaling. CKI1 is the first identified cytokinin responsive-histidine kinase that confers the ability in callus proliferation and shoot differentiation when overexpressed in Arabidopsis[11]. Arabidopsis histidine kinase 4 (*AHK4/CRE1/WOL*) encodes a sensor histidine kinase, which directly binds to cytokinin[24]. Two additional histidine kinases, AHK2 and AHK3, are homolog of AHK4 and function as positive regulators for cytokinin signaling[22]. However, the physiological roles of AHK2 and AHK3 in plant are not yet understood completely.

Recent studies have revealed that AHK3 functions as a histidine kinase and it transduces cytokinin signals across the membrane[22,24]. The *ahk3* mutant exhibited weak stimulation in cell proliferation by *t*-zeatin, a major cytokinin in plants[18], while normal to kinetin, a synthetic compound with cytokinin activity[8]. Although the *ahk3* responded normally to 6-benzyladenine (BA) during root elongation, it was less sensitive to *t*-zeatin in the adventitious root formation, comparing to the wild-type[8]. These differences suggest that the *ahk3* mutant may respond distinctly to different kinds of cytokinins.

Here, we demonstrate the *ahk3* mutant is less sensitive to *t*-zeatin during plant growth and development, and two specific ARRs are regulated AHK3-dependantly by *t*-zeatin. We also identified proteins down-regulated by *t*-zeatin by proteomic analysis.

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Materials and Methods

Plant materials and growth conditions

The T-DNA insertion mutant of AHK3 (*ahk3*) was obtained from SALK Institute Genomic Analysis Laboratory (California, USA), and confirmed by genomic-DNA PCR analysis using gene specific primers and T-DNA specific primers. *Arabidopsis thaliana* (Columbia ecotype) was used as a wild-type. Plants were grown on MS medium composed of 4.44 g/l MS salts (Duchefa, Netherlans), 2% (w/v) sucrose, 0.05% (w/v) 2-(*N*-morpholino) ethanesulfonic acid and/or 0.25% (w/v) agar (Sigma, USA) at pH 5.7. The sterilized seeds were plated and incubated at 4℃ for 2 days to ensure uniform germination, then transferred and incubated at 23℃ with a 16/8hr light/dark fluorescent illumination cycle.

Green callus formation assay and hormone treatment

Hypocotyl segments (explants) were excised from 14-day-old seedlings of wild-type and *ahk3* mutant. Explants were cultured on MS-agar plates containing 300 ng/㎖ *t*-zeatin and 300 ng/㎖ 1-naphtalene acetic acid (NAA). After incubation for 21 days, each representative callus were collected and photographed. For cytokinin treatment, wild-type and *ahk3* plants were grown on MS-agar plates containing *t*-zeatin at various concentrations for 8 days.

Cytokinin reduced leaf senescence assay

For senescence assays, seedlings were grown on MS-agar plates for 21 days and fully expanded leaves were excised from the seedlings. To induce senescence, leaves were floated on distilled water in plates supplemented with various concentrations of *t*-zeatin and incubated in growth chamber for 10 days. Chlorophylls were extracted and quantified spectrophotometrically as described previously[14].

Protein extraction and PEG fractionation

The samples were extracted with Mg/NP-40 buffer (0.5 M Tris-HCl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% (v/v) β-mercaptoethanol, and 1 mM phenylmethyl sulfonylfluoride) and fractionated with PEG 4000, following the method described by Kim *et al*[13]. After growing with 0, 0.01 and 1 μM *t*-zeatin for 8 days, each sample (2 g) was mixed with 20 ㎖ Mg/NP-40 buffer for 30 min and then centrifuged at 12,000 g for 15 min. The proteins in the supernatant were subjected into 15% (w/v) PEG 4000 for PEG fractionation. The mixture was incubated on ice for 30 min and then centrifuged at 12,000 g for 15 min. The supernatant was recovered and precipitated with acetone. The acetone precipitated proteins were solubilized in O'Farrell's buffer[19]. The protein concentration was measured by Bradford assay[2].

Two-dimensional gel electrophoresis (2-DE) and silver staining

2-DE was carried out according to O'Farrell[19] with some modifications following the protocols by Kim *et al* [13]. The proteins were first separated by isoelectric focusing (IEF) in a 15-cm IEF tube gel and then resolved in the second dimension by SDS-PAGE. The silver staining of 2-DE gels was according to Blum *et al*[1].

MALDI-TOF-MS (matrix-assisted laser desorption/ ionization-time-of-flight mass spectrometry) analysis and protein identification

Samples for the MALDI-TOF-MS analysis were extracted from silver stained spots as described previously[7]. The samples were digested overnight at 37 °C using 12.5 ng/ ㎕ trypsin (Promega, USA) in 25 mM ammonium bicarbonate (pH 8.0). In-gel digested peptide fragments were extracted three times from gel pieces using mixture of 5% (v/v) trifluoroacetic acid and 50% (v/v) acetonitrile. The solute materials including peptide fragments were dried by vacuum centrifugation. The peptide was dissolved into saturated α-cynao-4-hydroxycinnamic acid solution containing 5% (v/v) acetonitrile/ 2% (v/v) trifluoroacetic acid and loaded on a sample plate of MALDI-TOF-MS. Proteins were analyzed using MALDI-TOF-MS system (Reflex mode; Voyager-DE-STR, Perspective Biosystems, MA, USA). Spectra were calibrated using a bradykinin and neurotensin as internal standards. Peptide mass fingerprints were analyzed by MS-Fit (http://prospector.ucsf.edu/).

RT-PCR analysis

Samples were taken at indicated time points after treatment with 20 μM *t*-zeatin and total RNA was isolated using the Total RNA Extraction Kit (Promega, USA). Total RNA (2.5 ㎍) was used for first-strand cDNA synthesis after incubation at 65℃ for 10 min. cDNAs were synthesized in a volume of 20 ㎕ containing 5 x reverse transcriptase

buffer, 10 mM dithiothreitol, 1.5 μM poly (dT) primer, 0.5 mM dNTPs, and 1 unit of reverse transcriptase (Toyobo, Japan) at 37℃ for 1 h. The used gene specific primers for PCR reaction were shown in Table 1. Constitutively expressed *ACTIN-1* (At2g37620) was amplified in the same condition and used as an internal standard.

Results

Characterization of *ahk3* mutant

In order to understand the function of AHK3, we identified AHK3 T-DNA insertion mutants from the sequence-indexed SALK Institute Genomic Analysis Laboratory (SIGnAL) database, and two homozygous lines, *ahk3-5* (SALK_069269) and *ahk3-6* (SALK_067380), were isolated (Fig. 1A). Since cytokinins normally stimulate cell division and induce callus formation from explants[16], we examined the callus formation of *ahk3-5* and *ahk3-6*. The hypocotyl segments from wild-type, *ahk3-5*, *ahk3-6*, and *amp1* (a cytokinin overproducing mutant) were cultured on medium containing cytokinin (*t*-zeatin) and auxin (NAA). Less callus were formed in *ahk3-5* and *ahk3-6*, while the wild-type and *amp1* showed higher callus formation (Fig. 1B). We then examined the effect of exogenously supplied *t*-zeatin on the seedling growth of *ahk3-5*. The seedlings of *ahk3-5* (hereafter referred to as *ahk3*) were grown on vertical plate with or without *t*-zeatin. As shown in Figure 1C, no difference was observed between the mutant and wild-type in normal condition, whereas the mutant was slightly insensitive in the present of *t*-zeatin.

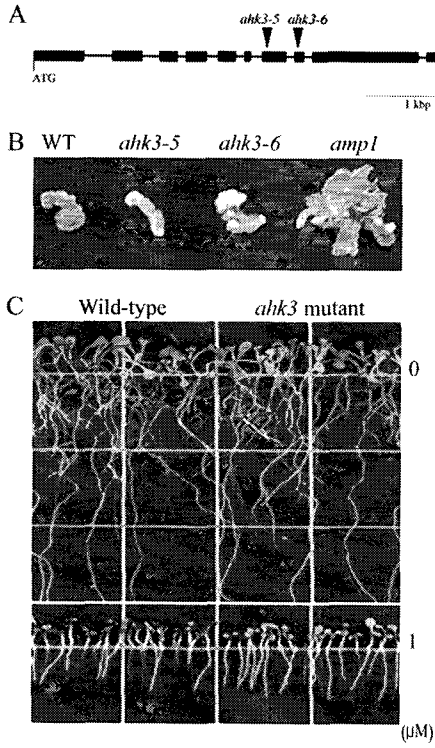

Fig. 1. Characterization of *ahk3* mutant. (A) Description of the *ahk3* T-DNA insertion alleles. Black boxes represent exons; horizontal bars, introns; and triangles, T-DNA insertion sites. (B) Induction of callus formation. The hypocotyl segments of wild-type and *ahk3* were cultured on MS medium containing 300 ng/㎖ *t*-zeatin and 300 ng/㎖ NAA. The *amp1* was used as a positive control. (C) Phenotype of *ahk3* seedlings. The wild-type and *ahk3* plants were grown on MS medium with or without 1 μM *t*-zeatin. The plants were photographed after 8 days.

Cytokinins inhibit leaf senescence in a variety of plant species[6]. We measured the chlorophyll contents to examine the effect of AHK3 mutation on senescence in the presence of *t*-zeatin (Fig. 2). After 10 days of dark-induced senescence, chlorophyll levels of wild-type and *ahk3* increased in dose-dependent manner. However, the increasing of chlorophyll contents in *ahk3* was smaller when compared to wild-type. These results suggest that *ahk3* mutant is *t*-zeatin insensitive.

Proteomic analysis of *ahk3* mutant

The *ahk3* exhibited less sensitivity to cytokinin in various plant growth and physiological processes. To identify differentially expressed proteins by *t*-zeatin treatment in *ahk3*, we performed proteomic analysis (Fig. 3). Six spots

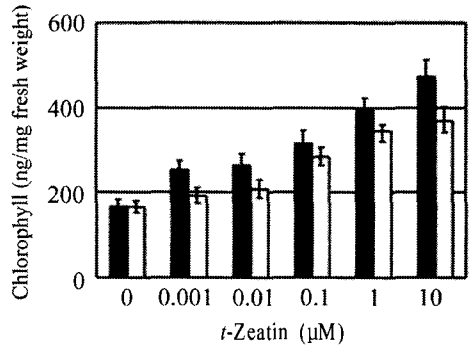

Fig. 2. Leaf senescence in response to *t*-zeatin. Fully expanded leaves were excised and floated on water supplemented with different concentrations of *t*-zeatin and incubated for 10 days in the dark. Chlorophyll contents were determined as described in Methods. Leaves from six plants per plate were examined three times at each concentration. Black bars represent wild-type; white bars, *ahk3*.

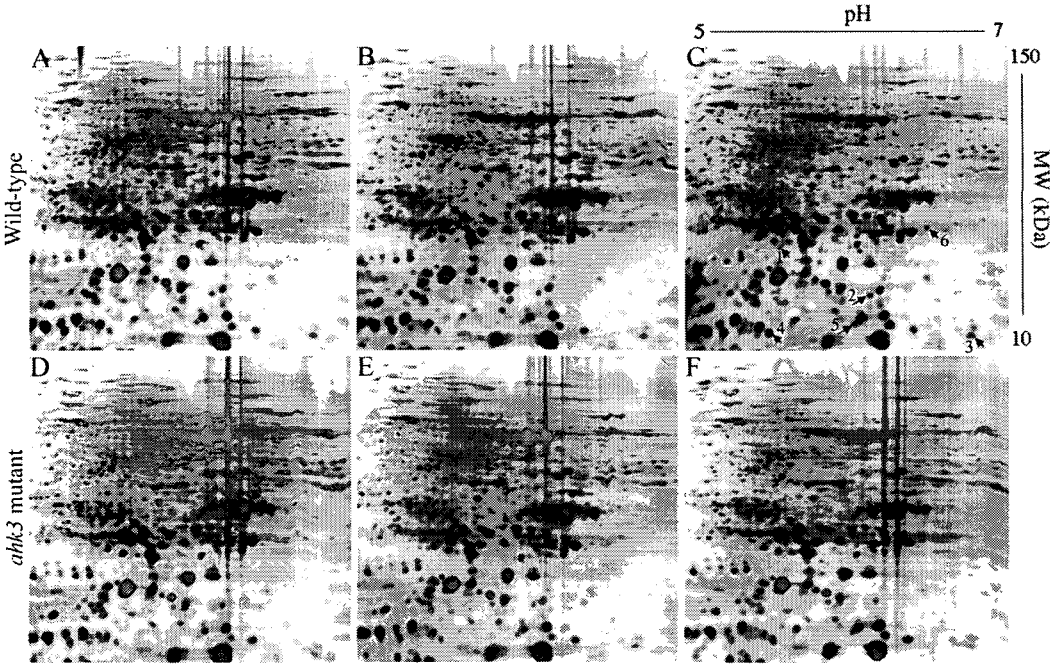

Fig. 3. The expression patterns of proteins in wild-type and *ahk3* by *t*-zeatin. Proteins were extracted from eight-day-old seedlings of wild-type (A-C) and *ahk3* (D-F). Plants were grown on plates with 0 μM (A and D), 0.01 μM (B and E), and 1 μM *t*-zeatin (C and F), respectively. Numbered spots (C) were excised, digested with trypsin, and analyzed by MALDI-TOF mass spectrometry.

from the 2D gel were selected for MALDI-TOF spectrometry and MS-Fit (http://prospector.ucsf.edu/) analysis. Three of them were up-regulated in wild-type but not in *ahk3* (Fig. 4A, Group Ⅰ) and the other three spots were induced both in wild-type and mutant (Fig. 4B, Group Ⅱ). The group Ⅰ were identified as eukaryotic translation initiation factor 5A-2, auxin binding glutathione *S*-transferase, and NDPK1, and the group Ⅱ were pollen allergen protein, major latex protein, and glutathione *S*-transferase Ⅱ, respectively (Table 2).

Transcriptional analysis of cytokinin primary-response gene family in *ahk3* mutant

We examine the effect of *t*-zeatin on the expression of A-type ARRs in *ahk3* (Fig.5). RT-PCR analysis was performed using gene specific primers for each gene (Table 1).

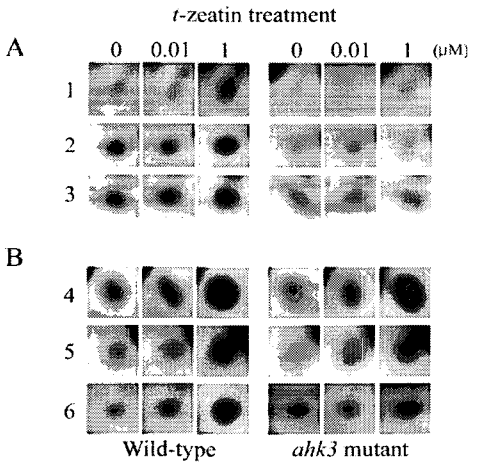

Fig. 4. Close-up of the region from the gels showing regulated proteins. The names of the proteins are shown in Table 2. The proteins in group Ⅰ (A) and in group Ⅱ (B).

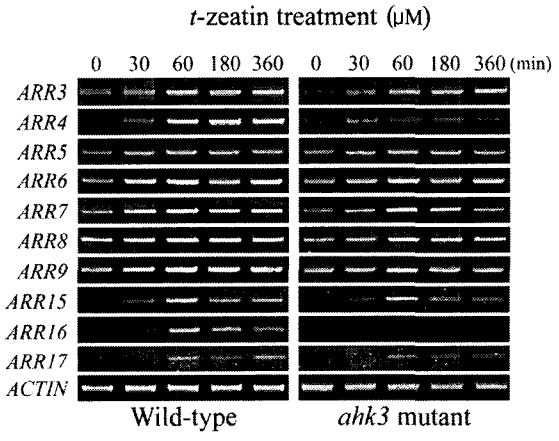

Fig. 5. RT-PCR analysis of A-type response regulators in *ahk3*. Total RNA was extracted from eight-day-old wild-type and *ahk3* which were treated with 20 μM *t*-zeatin at indicated time points.

Table 1. Primers used in PCRs

Name	Primer sequence		Size[a] (bp)	Tm[b] (℃)
	Forward primer	Reverse primer		
ARR3	5′-ATGGCCAAAGACGGTGGC-3′	5′-CTAAGCTAATCCGGGACTCCT-3′	696	53
ARR4	5′-ATGGCCAGAGACGGTGGT-3′	5′-CTAATCTAATCCGGGACTCC-3′	780	53
ARR5	5′-ATGGCTGAGGTTTTGCGT-3′	5′-TCAGATCTTTGCGCGTTTT-3′	555	53
ARR6	5′- ATGGCTGAAGTTATGCTACCG-3′	5′- TCAGATCTTTGCGCGTTTG-3′	489	53
ARR7	5′-ATGGCGGTTGGTGAGGTCA-3′	5′-CGTCAAAGTAGAGAAAAAAGGTTTTC-3′	623	55
ARR8	5′-ATGGTAATGGAAACAGAGTC-3′	5′-TCAGACCGAGGTTGTGATA-3′	678	50
ARR9	5′-ATGGGTATGGCAGCAGAATC-3′	5′-TCAGACAGCGGTTGCGATAC-3′	705	55
ARR15	5′-ATGGCTCTCAGAGATTTATC-3′	5′-TTAACCCCTAGACTCTAATT-3′	621	50
ARR16	5′-ATGAACAGTTCAGGAGGTTC-3′	5′-TTAGCTTCTGCAGTTCATGA-3′	495	50
ARR17	5′-ATGAATAAGGGCTGTGGAAG-3′	5′-TCAGCTTCTGCAATTTAAAA-3′	462	50
ACTIN-1	5′-ATGGCTGATGGTGAAGACATT-3′	5′-TCAGAAGCACTTCCTGTGAAC-3′	1134	53

The PCR reactions were performed 30 cycles.
[a]PCR product size.
[b]Annealing temperature of PCR reactions.

Table 2. The proteins identified by MALDI-TOF mass spectrometry

Spot No.	Identified protein	Accession No.	Sequence coverage (%)	pI	MW (kDa)
Group I					
1	Eukaryotic initiation factor 5A-2	AAM61392	45	5.6	17.14
2	Auxin binding glutathione S-transferase	P46422	20	5.9	24.13
3	NDPK1	P39207	30	6.3	16.50
Group II					
4	Pollen allergen protein	AAM65899	37	5.2	17.05
5	Major latex protein	AAM98319	37	5.9	17.47
6	Glutathione S-transferase II	CAA74639	22	6.3	23.55

Spots were classified in two groups of I and II. The proteins in group I were up-regulated in wild-type but not in *ahk3* by *t*-zeatin. The proteins in group II were induced in both wild-type and *ahk3* by *t*-zeatin.

Total RNA was extracted from wild-type and *ahk3* that were grown on MS liquid medium for 8 days and treated with *t*-zeatin at various time intervals. As shown in Figure 5, transcripts of A-type ARRs were rapidly induced by *t*-zeatin in wild-type, and most of them showed similar expression patterns in *ahk3*, except *ARR4* and *ARR16*. The induction of *ARR4* and *ARR16* by *t*-zeatin was markedly reduced in *ahk3*. This result suggests that ARR4 and ARR16 might be related to AHK3-mediated cytokinin signaling.

Discussion

Cytokinins can be divided into two types, natural adenine-type and synthetic phenylurea-type. The *t*-zeatin, BA, and kinetin are adenine-type cytokinins, and *t*-zeatin is the most abundant and active cytokinin in plants[16]. We demonstrated that the *AHK3* deficient mutant is less sensitive to *t*-zeatin specifically in callus formation, seedling growth, and senescence whereas the *ahk3* was known to be normal to kinetin and BA in callus formation and root elongation[8,18]. These results suggest that *ahk3* have distinct sensitivities in response to different kinds of cytokinins.

Proteomic analysis of *ahk3* has led to identify several downstream components. Six proteins in wild-type were induced by *t*-zeatin, while three of them were down-regulated in *ahk3*. The three proteins are identified as eukaryotic translation initiation factor 5A (eIF 5A), auxin binding glutathione *S*-transferase (auxin binding GST), and nucleoside-diphosphate kinase 1 (NDPK1), respectively. The eIF 5A is thought to be present in all eukaryotic cells[10] and functions in cell division and cell death via facilitation of mRNA translation[26]. Arabidopsis has three isoforms of eIF 5A, and two of them were examined to be involved in cell death using animal system[26], but the roles in plant

remain unclear. GSTs are one of the superfamilies in plant that are regulated by wide range of biotic and abiotic stresses and are working in detoxifying oxidative-stress metabolites[5]. A GST identified from our study has been reported to be localized in plasma membrane vesicles and bind to auxin[25]. However, the roles in plants are largely unknown. NDPK is one of the multifunctional proteins that regulate cell proliferation, development, and differentiation[20]. Plant NDPKs are involved in UV-B signaling, phytochrome responses, and stress responses[3,17,26]. Although the proteins identified were induced by *t*-zeatin in AHK3 dependent manner, the roles of these proteins in cytokinin signaling have not been investigated yet.

Even though the AHK3 is a close homolog of AHK4, the features in root growth, leaf development, formation and maintenance of meristem activity are different[18]. Kiba *et al*[12] proposed that ARR15 and ARR16 are involved in the AHK4-mediated signaling pathway in root. In our study, we observed that the expressions of ARR4 and ARR16 in *ahk3* were markedly decreased by *t*-zeatin treatment. Thus, we propose that ARR4 and ARR16 are linked to AHK3-mediated cytokinin signaling, which is different from AHK4. Further studies are required to verify this.

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초록 : 애기장대 histidine kinase 3 (AHK3)의 특성과 결손돌연변이체인 *ahk3*의 프로테옴 분석

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Histidine kinase는 식물의 신호전달기작에서 매우 중요한 역할을 한다. 본 연구에서는 애기장대 histidine kinase 3 (AHK3)의 식물체내에서의 기능을 조사하였으며 이 유전자의 결손 돌연변이체인 *ahk3*에 *trans*-zeatin (*t*-zeatin)을 처리하여 유전자와 단백질의 발현양상을 분석하였다. *ahk3*는 야생형 식물체에 비하여 캘러스 형성, 유모의 성장, 잎의 노화과정에서 *t*-zeatin에 대한 감수성이 줄어들었다. 프로테옴 분석 결과 eukaryotic translation initiation factor 5A-2, auxin binding glutathione S-transferase, NDPK1 등은 야생형의 애기장대에서는 *t*-zeatin에 의하여 발현이 증가하는 반면 *ahk3*에서는 증가하지 않는 것으로 나타났다. 또한 cytokinin 처리에 의하여 발현이 증가하는 것으로 보고된 A-type response regulator들 중에서 *ARR4*와 *ARR16*의 발현양이 *ahk3*에서는 현저하게 감소하는 것으로 나타났다. 이러한 결과들은 AHK3가 cytokinin 신호전달기작에서 매우 중요한 역할을 하며, 프로테옴 분석에 의하여 동정된 단백질들과 ARR4, ARR16은 AHK3에 의해 매개되는 cytokinin 신호전달과정에서 중요한 역할을 할 것으로 생각된다.