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A Crucial Role for Cytokinins in Pea ABR17-mediated Enhanced Germination and Early Seedling Growth of *Arabidopsis thaliana* under Saline and Low-temperature Stresses

Sanjeeva Srivastava,[1] R. J. Neil Emery,[2] Muhammad H. Rahman,[1] and Nat N. V. Kav[1]*

[1]*Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton Alberta T6G 2P5, Canada;* [2]*Department of Biology, Trent University, Peterborough, Ontario K9J 7B8, Canada*

Abstract

The role of cytokinins (CKs) in mediating the previously observed ABR17-mediated enhancement of germination of *Arabidopsis thaliana* under salinity and low-temperature stresses has been evaluated. We determined the endogenous concentrations of CK in the three transgenic and wild-type seedlings, which indicated that the transgenic seedlings had higher endogenous concentrations of CK. Furthermore, the relative levels of expression of *ABR17* cDNA and the primary CK response gene, *ARR5*, were evaluated in the transgenic and wild-type seedlings by quantitative real-time polymerase chain reaction (RT-PCR). Our results indicated that two of the three independently derived transgenic plants possessed higher levels of *ABR17* transcripts, which correlated well with increased *ARR5* expression, further supporting a possible role for CK in mediating the observed phenomenon. In addition, the exogenous application of various CKs on the germination of wild-type *A. thaliana* under these abiotic stress conditions enhanced its germination. Finally, the ribonuclease (RNase) activity of the pea ABR17 protein was also demonstrated after expression of its cDNA in *Escherichia coli*, purification of the recombinant protein, and *in vitro* RNase assays. Our findings are discussed within the context of ABR17-mediated enhancement of endogenous CK concentrations, the involvement of CKs in germination under abiotic stress, as well as the role of the RNase activity of ABR17 protein in mediating the observed effects.

Key words: *Arabidopsis*; *ABR17*; *AtARR5*; Cytokinin; Pathogenesis-related PR 10; Ribonuclease; tRNA

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**Corresponding author; e-mail:* mail: nat@ualberta.ca

Introduction

Pathogenesis-related (PR) proteins are produced by plants in response to various abiotic and biotic stresses and include the intracellular pathogenesis-related (IPR) protein family also known as PR 10 proteins (van Loon and van Strien 1999). PR 10 proteins are encoded by multigene families, and are small (15–18 kDa) acidic proteins that are resistant to proteases (Warner and others 1994). Some PR 10 proteins are developmentally regulated, and they are constitutively expressed in different plant organs in many different species, which suggest their possible involvement in general plant growth and development (Sikorski and others 1999; Wu and others 2002). Although the expression of *PR 10* genes under various stress conditions, their accumulation in different plant organs, and their physicochemical properties are well characterized, the precise biological function of PR 10 proteins in plant growth and development are as yet unknown.

The activities of PR 10 proteins are also unclear, although, based on high amino acid sequence similarities between PR 10 proteins and RNases, it has been suggested that they may be RNases (Moiseyev and others 1994). In fact, PR 10 proteins from various species have been demonstrated to possess RNase activity (Srivastava and others 2006a and references therein). Some PR 10 proteins have also been reported to interact with many ligands, including cytokinins (CKs), brassinosteroids, fatty acids, and flavonoids, and these interactions have led to the suggestion that all PR 10 proteins may not be RNases (Fujimoto and others 1998; Mogensen and others 2002; Markovic-Housely and others 2003). Based on weak sequence homology and secondary-structure prediction, the cytokinin-specific binding proteins (CSBPs) have also been included in the PR 10 class (Fujimoto and others 1998). In addition, Mogensen and others (2002) as well as Markovic-Housley and others (2003) have suggested a general plant hormone carrier function for PR 10 proteins during plant defense response to pathogens, as well as during normal growth and developmental processes (Pasternak and others 2005).

In peas, PR 10 proteins are encoded by a family of genes comprised of five known members: PR 10.1–10.3 (also known as Drr49a–c), and pea abscisic acid (ABA)-responsive proteins (ABR) 17 and 18 (also known as PR 10.4 and 10.5, respectively) (Tewari and others 2003). Pea *ABR17* and *18* are expressed during seed development and are induced by exogenous application of ABA (Iturriaga and others 1994). Recently we demonstrated that the level of ABR17 protein is increased in the roots of salinity-stressed pea plants, which suggested a potential role for pea ABR17 in mediating plant responses to salinity and other abiotic stresses (Kav and others 2004). This hypothesis was tested using the model plant *Arabidopsis thaliana*, and it was confirmed that *ABR17* is able to enhance germination and early seedling growth under saline stress conditions as well as low-temperature stress conditions (Srivastava and others 2006b). We have also demonstrated that the constitutive expression of another pea *PR 10* cDNA (*PR 10.1*) in *Brassica napus* enhanced germination and early seedling growth under saline conditions (Srivastava and others 2004), which appeared to correlate well with an elevated level of endogenous CKs (Srivastava and others 2006a).

In the present study we have investigated the involvement of CKs in mediating the enhanced germination and early seedling growth of ABR17 transgenic *A. thaliana* by determining the endogenous CK concentrations and the expression of the primary CK-responsive gene *ARR5* in the transgenic plants. We have also demonstrated that the germination of wild-type *A. thaliana* under abiotic stress is enhanced by the exogenous application of various CKs. The RNase activity of recombinant ABR17 has also been demonstrated for the first time, and our findings are discussed within the context of a role for CKs in enhancing germination and early seedling development.

Materials and Methods

Plant Material and Growth Conditions

Three *A. thaliana* lines (6.9, 14.9, and 25.20) constitutively expressing pea *ABR17* cDNA have been previously described (Srivastava and others 2006b). Seeds from transgenic, homozygous plants of T2 generation and the wild-type (ecotype WS) were germinated and grown in plastic trays containing Metro Mix® 290 (Grace Horticultural products, Ajax, Ontario, Canada) in the greenhouse (22°C day/18°C night, 16 h photoperiod) and fertilized with a complete fertilizer (Peters^{TM}20-20-20, Plant Products, Bramalea, Ontario, Canada) containing micronutrients. The plants were allowed to grow to maturity and phenological observations were made. Additionally tissue from 2-week-old plants was also collected for phytohormone analysis. Wild-type and transgenic (6.9, 14.9, 25.20) *A. thaliana* seedlings were also grown on Petri dishes to

obtain sterile tissue for the isolation of RNA and for phytohormone analysis. For this purpose, seeds were rinsed for 1 min with 70% ethanol and surface sterilized with 20% bleach for 15 min, after which they were washed four times (5 min each) with sterile deionized water to remove the bleach. The sterilized seeds were placed on solid ½ strength MS medium containing 1.5% sucrose, 0.8% agar, pH 5.7, for 2 weeks, after which the seedlings were harvested.

Determination of Endogenous CK Concentrations

Endogenous concentrations of various phytohormones in 2-week-old *A. thaliana* seedlings (wild-type and the transgenic lines) grown in the greenhouse as well as on solid ½ strength MS medium, as described earlier (Srivastava and others 2006a), were measured. Tissue harvested from either greenhouse grown plants or aseptic cultures was flash frozen in liquid nitrogen, lyophilized, and used for phytohormone analysis. Cytokinins were extracted, purified, and quantified by an isotope dilution assay under conditions established by Emery and others (1998, 2000) and Ferguson and others (2005). Deuterated CKs, [$^2H_6$]iP, [$^2H_6$][9R]iP, trans-[$^2H_5$]Z, [$^2H_3$]DZ, trans-[$^2H_5$][9R]Z, [$^2H_3$][9R]DHZ, [$^2H_6$][9R-MP]iP, and [$^2H_6$][9R-MP]DHZ (OlChemIm Ltd, Olomouc, Czech Republic) were added as quantitative internal standards. Nucleotides were converted to nucleosides for quantification. Purified CK were separated and analyzed by (LC-(+)ESI-MS/MS) using a Waters 2680 Alliance HPLC system (Waters, Milford, USA) linked to a Quattro-LC triple quadrupole MS (Micromass, Altrincham, UK).

Quantitative Real-time (RT)-PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was performed to investigate the relative levels of expression of the *ABR17* and *Arabidopsis* response regulator (*AtARR*) 5 using the expression of actin as the control. Polymerase chain reaction primer sets for RT-PCR were designed using the Primer Premier 3 software (Applied Biosystems Inc., CA, USA) to generate PCR products of approximately 70–80 bp. The primer sets used in these experiments for the various genes are shown in Table 1. Total RNA was extracted using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) from pooled, 2-week-old seedling tissue and treated with RNase-free DNase (Qiagen) to remove contaminating genomic DNA. First strand cDNA was synthesized by reverse transcription of total RNA (50 ng) using the iScript cDNA synthesis kit (Bio-Rad, CA, USA). The PCR reaction (20 µl) contained 2 µl of 5× diluted cDNA as template, 22.5 pmol of each primer, 5 pmol probe, and 1× TaqMan Universal PCR Master Mix (Roche, New Jersey, USA). The real-time quantification was performed in a ABI prism 7700 Sequence detector (Applied Biosystems) using the SNP RT template program. The comparative relative expression was determined using the delta-delta method employing the formula relative expression = $2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$ (Livak and Schmittgen 2001), where Ct refers to the threshold cycle. The level of expression in the wild-type was considered to be 1, and the relative expression in the transgenic lines was normalized against that level. The reactions were performed in duplicate, and the experiments were repeated at least three times.

Table 1. Primer Sequences for Overexpression and Quantitative Real-time PCR Analysis

Gene	Primer pairs and probe used in real-time PCR
Ps*ABR17*	Forward; 5′-AAATGGAGGTCCAGGAAC CAT-3′
	Reverse; 5′-AGCACATAGTTGGTTTTTCC ATCTT-3′
	Probe; 5′-AGAAGCTATCCATTCTT-3′
At*Actin*	Forward; 5′-GCCATTCAGGCCGTTCTTT-3′
	Reverse; 5′-ATCGAGCACAATACCGGTTGT-3′
	Probe; 5′-TCTATGCCAGTGGTCG-3′
At*ARR5*	Forward; 5′-TTCATTAGCATCACCGAA ACTTCT-3′
	Reverse; 5′-CGATGAACTTCCGATCAACCA-3′
	Probe; 5′-TTCTTGCTGTTGATGATAG-3′

Exogenous Application of CKs

Two different approaches were used to test the ability of various CKs to enhance germination and early seedling growth of wild-type *A. thaliana* under conditions of stress. In one experiment, wild-type *A. thaliana* seeds were surface sterilized as described previously and placed on medium containing various concentrations (1, 5, and 25 µM) of CKs (mixed isomer-zeatin, trans-zeatin riboside, BAP, 2-iP or kinetin) with or without 150 mM NaCl in 25 × 100 mm Petri dishes under continuous light at 21° ± 1°C or 10°C. In the second approach, surface sterilized wild-type seeds were imbibed in 25 µM aqueous solutions of different

CKs [mixed isomer-zeatin, *trans*-zeatin riboside, 6-benzyl amino purine (BAP), 6-(γ,γ-Dimethyl allyl amino) purine (2iP) or kinetin] for 4 h and washed 4 times (5 min each) with sterile DW, after which they were placed on plates containing 150 mM NaCl, but without CK. The plates containing the seeds were then kept at 21° ± 1°C or 10° ± 1°C under continuous light. For both types of experiments, at least 5 plates per treatment (14 seeds per plate) were used, and seedlings were grown for 2 weeks. Each experiment was repeated at least three times, and the percentage of germinated seeds calculated for each and the data statistically analyzed as described below.

Overexpression, Purification of Pea *ABR17* in *E. coli* and RNase Activity Determination

Pea *ABR17* cDNA was amplified using two specific primers; Forward 5′-gtg gtc gca tat gga aaa ttt gta ctt tca agg tat ggg tgt ctt tgt ttt tga tga tga ata c-3′ and Reverse 5′-tat ata gct cga gtt agt aac cag gat ttg cca aaa cgt aac c-3′. The forward primer possessed an *Nde* I (underlined) as well as the rTEV protease recognition sequence (bold), and the reverse primer contained an *Xho* I recognition site (underlined). Polymerase chain reaction amplified products were digested with *Nde* I and *Xho* I (New England Biolabs, Ontario, Canada) and ligated into similarly digested pET28a bacterial expression vector (Novagen, WI, USA) to generate a hexahistidine-tagged fusion protein upon expression. The overexpression, purification of pea *ABR17* and RNase activity determinations were performed using the methods previously described for the pea PR 10.1 protein (Srivastava and others 2006a).

Statistical Analysis

All statistical analyses were performed using the mixed model procedure of SAS version 8e (Statistical Analysis System, 1985). Analysis of variance was performed using a mixed model and least square means were separated using a Pdiff option for significant ($p < 0.05$) fixed effects.

Results

Appearance of *ABR17* Transgenic *A. thaliana* Plants

The appearance of wild-type and three independently derived transgenic lines (lines 6.9, 14.9, and 25.20) at various stages of growth and development is shown in Figure 1 (A–E). It is clear from the images presented in Figure 1 that the transgenic lines are developmentally further ahead of the wild-type at all stages. Both the wild-type and transgenic lines started to bolt during the third week, but it was apparent that bolting was earlier in all the transgenic lines, in particular line 6.9, as compared to the wild-type counterpart (Figure 1C). Time (days) to opening of the first flower was also earlier (at least 24–48 h) in transgenic lines as compared to the wild-type. The number of lateral branches originating from the primary inflorescence were counted, and it was observed that the average number was significantly greater ($p < 0.01$) in the transgenic lines. The number of siliques in each plant was counted when the stem turned brown (~ 6 weeks), and the number of mature siliques was found to be higher in the transgenic lines. Similarly, when the number of siliques in the various genotypes was determined, it was observed that transgenic line 6.9 possessed a significantly ($p < 0.05$) higher number than the other two transgenic or the wild-type lines (Figure 2B). These results indicate that introduction of pea *ABR17* cDNA has positive effects on enhancing growth and development of transgenic *A. thaliana* plants.

Endogenous Concentration of CKs in *ABR17* Transgenic *A. thaliana*

The endogenous levels of various types of CKs and their nucleoside and nucleotide precursors in wild-type and *ABR17* transgenic *A. thaliana* were determined, and the results are shown in Table 2. In 2-week-old seedling tissue obtained from MS plates, two of the three transgenic lines (6.9 and 25.20) showed an increase in the endogenous concentration of *cis*-Z, *trans*-Z, *cis*-[9RMP]Z and *trans*-[9RMP]Z, whereas the concentrations of these CKs were not different in the other transgenic line (14.9) when compared to the wild-type (Table 2). A dramatic increase in the levels of *trans-ZR* and a moderate increase in [9R]iP were observed in line 25.20 (tissue from MS plate), whereas a similar increase in the concentration of these forms of CK was not observed in the other two transgenic lines or in the wild-type (Table 2). In addition, the concentrations of iP in tissues of MS-grown transgenic lines 6.9 and 25.20 were also higher than that of wild-type seedlings (Table 2).

In 2-week-old soil grown plants it was observed that the concentration of *cis*-Z was higher in all of the three transgenic lines, whereas only line 25.20 demonstrated an increase in concentration of *cis*-[9RMP]Z (Table 2). The concentration of *trans*-Z

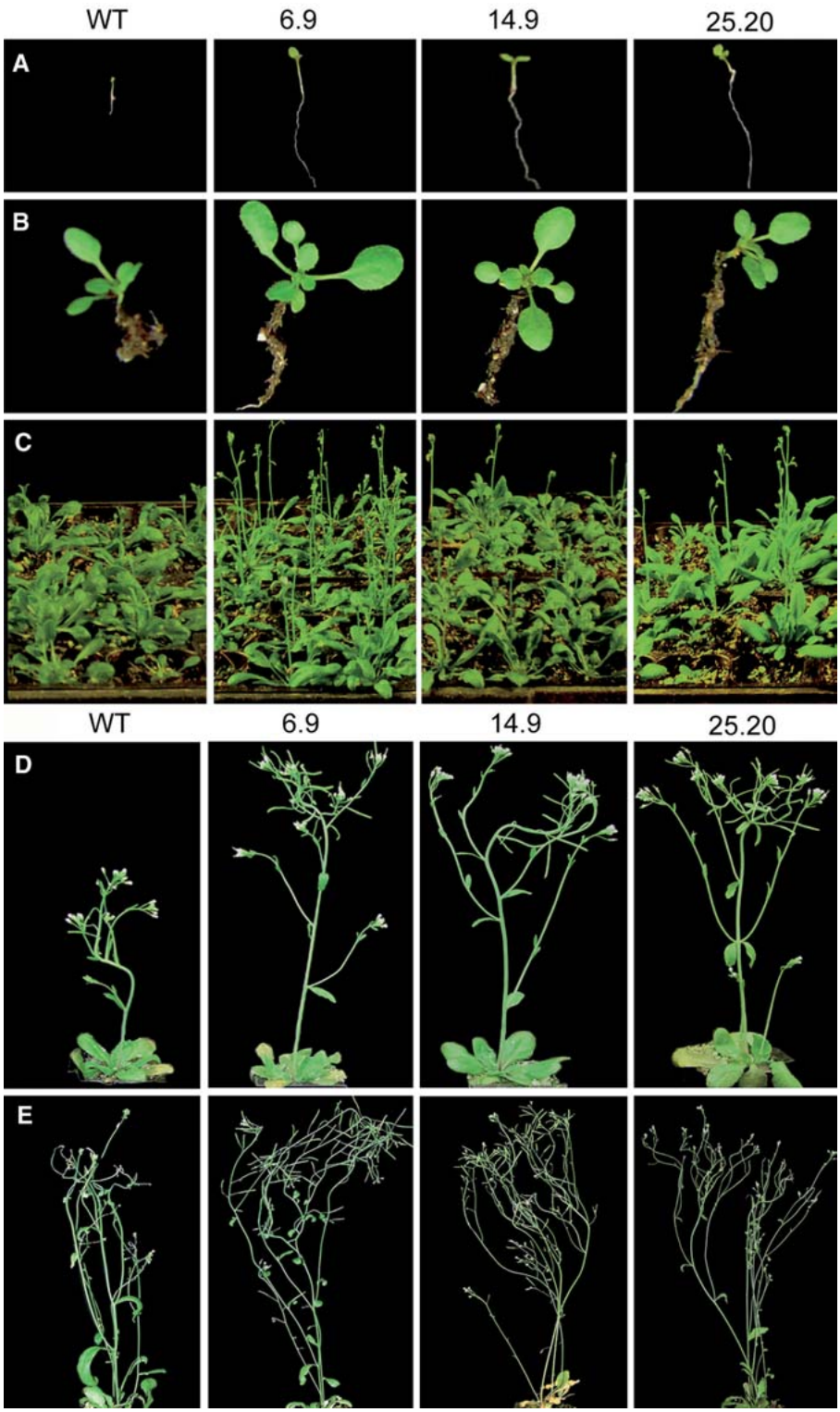

Figure 1. Enhanced germination and overall growth of wild-type and transgenic (6.9, 14.9 and 25.20) *A. thaliana* constitutively expressing pea *ABR17* cDNA. Appearance after (A) 5 days (B) 2 weeks (C) 3 weeks (D) 4 weeks and (E) 5 weeks.

was not different in any of the four genotypes, whereas *trans*-ZR and *trans*-[9RMP]Z showed a decrease in all three transgenic lines, which contributed to the overall reduction in the concentrations of free CK bases in all the transgenic lines and the reduction in concentration of CK nucleotides in lines 14.9 and 25.20 (Table 2). We should emphasize that CKs were measured only at a single developmental stage (immediately prior to bolting), and this may not have captured all the differences

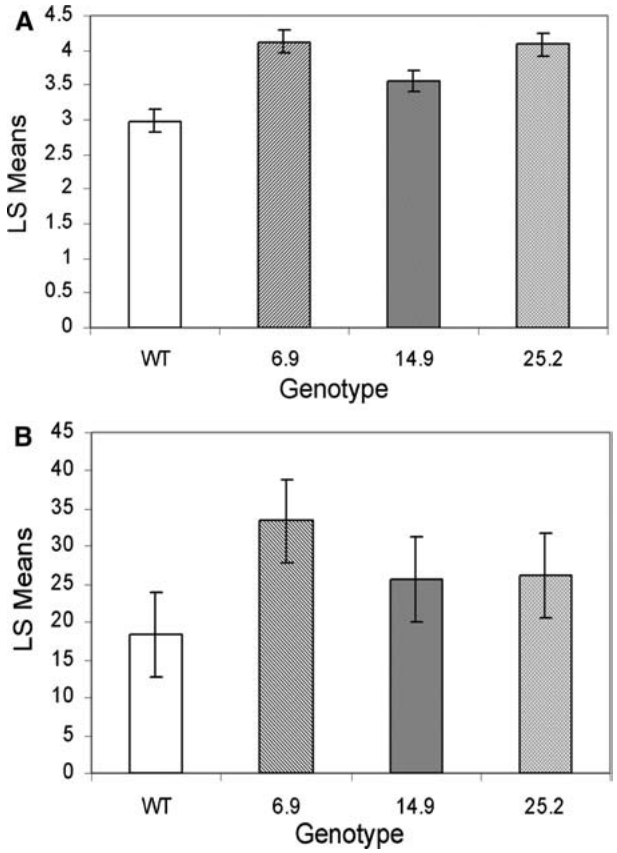

Figure 2. Increased lateral branching and siliques in *ABR17*-transgenic *A. thaliana*. (A) Lateral branches and (B) siliques in wild-type and three *ABR17* transgenic lines (6.9, 14.9 and 25.20).

between the three transgenic lines and the wild-type. However, it is clear that in tissue from MS-grown *A. thaliana* seedlings, significant increases in specific CK forms are observed in the transgenic lines when compared to the wild-type.

Analysis of *ABR17* and *AtARR5* Gene Expression

To further investigate the role of *ABR17* in mediating the observed phenotype of the *ABR17* transgenic *A. thaliana* seedlings, the relative levels of expression of the pea *ABR17* gene in the three transgenic lines (6.9, 14.9, and 25.20) were assessed using quantitative RT-PCR. In addition, to further examine our hypothesis that the enhanced germination under abiotic stress due to *ABR17* expression is mediated, at least in part, by an increase in CKs, we also investigated the relative expression of the CK primary response gene *AtARR5* using Q-RT-PCR. Among the transgenic lines, 6.9 had the highest level of *ABR17* expression, and the other two transgenic lines had comparable levels (Table 3). The levels of expression of the primary CK response regulator *AtARR5* also correlated well with that of *ABR17*, with all transgenic lines showing increased levels of the *AtARR5* transcript (Table 3). Furthermore, the level of *AtARR5* expression was also the highest in line 6.9, which also correlates with the line exhibiting the highest level of *AtABR17* expression (Table 3). The other two transgenic lines had approximately half the level of *AtARR5* expression as line 6.9.

Effects of Exogenous CK on Germination of *A. thaliana* Under Salinity and Cold Stress

To demonstrate the direct involvement of CKs in enhancing the germination of *A. thaliana* under abiotic stress, we investigated the effects of various CKs on the germination and subsequent development of wild-type *A. thaliana* by two different approaches. In the first approach, where various CKs were included in the culture medium at a concentration of 5 μM, the percentage of seeds germinating in the presence of 150 mM NaCl at RT was significantly ($p < 0.05$) higher on day 5 and remained high until the conclusion of the 14-day experiment in the presence of CKs compared to control plates with no exogenous CK (Figure 3A). The appearance of the seedlings after the 14-day experiment for this treatment is shown in Figure 3B, and it is evident that the treated seedlings are developmentally more advanced than the untreated ones. At the end of the 14-day period, it was also evident that the CKs zeatin, benzylamino purine (BAP), isopentenyl adenine (2 iP), and kinetin were able to enhance the germination of *A. thaliana* in the presence of 150 mM NaCl, significantly (Figure 3A). Even though *trans*-ZR was able to enhance germination under these conditions, the extent of this enhancement was lower than the other CKs tested (Figure 3A).

The ability of CKs to enhance germination in the presence of 150 mM NaCl at 10°C was also evaluated, and the results are shown in Figure 3C–D. Preliminary experiments at this temperature showed that 1 μM CK concentration was the most effective in enhancing the germination when included in the growth medium (data not shown). In the presence of a 1 μM concentration of various CKs and 150 mM NaCl at this lower temperature, the percentage of seeds that germinated was significantly ($p < 0.05$) higher than in the absence of CKs, and this enhancement was apparent on day 6 and continued until day 14 (Figure 3C). All the

Table 2. Endogenous Concentrations of CK in Wild Type and ABR17 Transgenic *A. thaliana*

Cytokinins	Quantity (pmol gDW^{-1})							
	Wild type (mean ± SE)		6.9 (mean ± SE)		14.9 (mean ± SE)		25.20 (mean ± SE)	
	MS plates grown plants (n = 2)	Soil grown plants (n = 3)	MS plates grown plants (n = 2)	Soil grown plants (n = 3)	MS plates grown plants (n = 2)	Soil grown plants (n = 3)	MS plates grown plants (n = 2)	Soil grown plants (n = 3)
trans-Z	25.1 ± 3.3	46.7 ± 0.8	40.6 ± 7.0	48.2 ± 0.8	28.0 ± 0.1	41.1 ± 2.0	87.2 ± 0.3	44.2 ± 1.6
cis-Z	66.5 ± 1.3	23.6 ± 0.9	87.0 ± 4.4	42.6 ± 0.9	69.8 ± 1.4	43.7 ± 1.0	105.8 ± 2.7	46.8 ± 1.8
IP	2.6 ± 0.1	2.0 ± 0.1	5.9 ± 1.2	5.2 ± 0.8	2.2 ± 0.1	2.5 ± 0.0	38.5 ± 0.6	3.1 ± 0.1
trans-[9R]Z	92.5 ± 1.3	268.9 ± 1.9	88.3 ± 7.0	213.3 ± 1.2	63.5 ± 0.3	207.9 ± 1.6	262.2 ± 0.5	151.4 ± 5.2
cis-[9R]Z	11.5 ± 0.8	15.1 ± 0.3	9.8 ± 0.7	18.3 ± 0.9	8.9 ± 0.3	11.0 ± 0.6	14.7 ± 1.5	12.6 ± 0.5
[9R]DHZ	0.0 ± 0.0	20.6 ± 0.6	0.0 ± 0.0	20.1 ± 0.4	0.0 ± 0.0	22.1 ± 0.5	22.8 ± 0.1	20.8 ± 2.1
[9R]iP	54.7 ± 1.3	95.5 ± 0.6	36.6 ± 2.1	95.6 ± 0.5	38.0 ± 0.4	79.6 ± 1.7	65.2 ± 2.2	86.5 ± 1.5
FB total	252.9 ± 0.3	472.4 ± 3.0	268.2 ± 21.1	443.3 ± 1.4	210.4 ± 2.4	408.0 ± 4.7	596.3 ± 1.5	365.3 ± 2.9
trans-[9RMP]Z	203.5 ± 24.7	1060.5 ± 27.5	315.8 ± 0.8	1058 ± 13.2	209.3 ± 1.9	938.5 ± 22.1	460.8 ± 10.8	715.7 ± 72.5
cis-[9RMP]Z	49.8 ± 11.4	56.9 ± 1.19	75.5 ± 13.4	82.1 ± 2.9	56 ± 1.1	41.7 ± 2.2	93.2 ± 3.5	36.7 ± 4.2
[9RMP]DHZ	19 ± 2.8	37.3 ± 1.09	21.4 ± 8	30.8 ± 2.8	25.6 ± 2.6	36.6 ± 0.9	28.4 ± 4.3	34.2 ± 0.6
[9R]iP	273 ± 1.3	518.8 ± 6.5	342.7 ± 5.6	561.9 ± 11.1	234.2 ± 0.4	482.9 ± 0.3	298 ± 4.7	597.9 ± 9.8
NT total	545.3 ± 17.5	1673.5 ± 32.5	755.4 ± 11.7	1732.9 ± 22.5	525 ± 1.4	1500 ± 24	880.4 ± 7	1384.4± 83.7
Total CK	798.2 ± 17.8	2145.8 ± 35.5	1023.6 ± 32.7	2176.1 ± 21.3	735.5 ± 3.7	1907.7 ± 28.7	1476.7 ± 8.5	1749.7 ± 80.5

Table 3. Gene Expression Analysis (Relative Fold Expression)

Gene	Relative fold expression[a]			
	Wild-type	6.9	14.9	25.20
Ps*ABR17*	1 ± 0	3115 ± 1143	961 ± 278	1128 ± 301
At*ARR5*	1 ± 0	9 ± 1	4.3 ± 0.5	4.1 ± 0.2

[a]*Gene expression in the wild-type was considered to be 1, and the relative expression in the transgenic lines was normalized against the wild-type expression level.*

types of CKs tested were equally effective, with perhaps BAP being the most effective and *trans*-ZR being the least when observed at the conclusion of the 14-day experiment (Figure 3D). The appearance of the seedlings at the conclusion of the experiment (Figure 3D) also supports the above conclusions.

In the second approach when wild-type seeds were imbibed in 25 μM aqueous solutions of different CKs and subsequently placed on ½ MS plates in the presence of 150 mM NaCl at RT, a significant increase in the percentage of germinated seeds was observed even on day 3 and remained high until the conclusion of the experiment on day 14 (Figure 4A). The same trends were observed with both approaches, which demonstrated a significant enhancement in germination by exogenous application of various CKs. The notable difference between the two types of approaches of administering the CKs was that in the first approach zeatin was most effective (Figure 3A) and in the second approach 2 iP was most effective (Figure 4A); with both approaches, *trans*-ZR was least effective. Once again, as can be seen from the appearance of the seedlings at the conclusion of the 14-day experiment, seeds that were treated with CKs are more advanced than the untreated controls (Figure 4B).

When the seeds were imbibed in CK solutions as described earlier and placed on plates containing 150 mM NaCl at 10°C, a similar enhancement of germination of the treated seeds was observed (Figure 4C and D). We were able to observe this enhancement as early as day 4, and it continued until the end of the 14-day experiment, at which time all the CKs tested were effective in enhancing germination significantly ($p < 0.05$). Similar to what we observed in this type of CK treatment at RT, *trans*-ZR was the least effective in enhancing the percentage of germinated seeds at 10°C (Figures 4C and D).

RNase Activity of Pea *ABR17* Protein

To determine whether pea *ABR17* is also an RNase we overexpressed the cDNA in *E. coli* and purified the recombinant protein using metal-chelation affinity chromatography as previously described for pea PR 10.1 (Srivastava and others 2006a). The recombinant protein was expressed in significant amounts upon induction with IPTG, and we were successful in purifying the protein with or without the histidine tag (Figure 5A). Both forms of the protein (with or without the histidine tag) were tested for RNase activity using in-solution and in-gel activity assays (Srivastava and others 2006a). It is clear from the results shown in Figure 5B that both forms of the protein were able to degrade RNA isolated from pea and canola. As described earlier, an in-gel assay was also performed to investigate whether the recombinant *ABR17* protein is an RNase (Figure 5C). Proteins with RNase activities upon separation in this gel system containing yeast tRNA produce clear regions at the expected molecular weights (Srivastava and others 2006a). As can be observed from the gel shown in Figure 5C, we observed a clear region against a blue background precisely at the molecular weight of the recombinant protein with and without the histidine tag. The identity of the protein was also verified as pea *ABR17* by excising the band from a sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE) study performed under identical conditions but without the yeast tRNA and tandem mass spectrometry (data not shown).

Discussion

In the present study we investigated the mechanisms underlying previously reported pea *ABR17*-mediated enhancement of germination of *A. thaliana* under saline and low-temperature stress conditions. Based on the ability of another pea PR 10 protein (PR 10.1) to enhance endogenous concentrations of CKs (Srivastava and others 2006a), we hypothesized that some of the *ABR17*-mediated effects in *A. thaliana*, in particular the enhanced germination and early seedling growth under abiotic stress, could also be mediated through an elevation of endogenous CKs. This hypothesis was tested by determining the endogenous concentrations of various forms of CKs in tissue obtained from seedlings grown on MS plates as well as in soil. Tissue from 2-week-old seedlings grown on MS plates was used, because our earlier study had demonstrated that transgenic seedlings grown in this manner were significantly more tolerant of abiotic stresses (Srivastava and others 2006b).

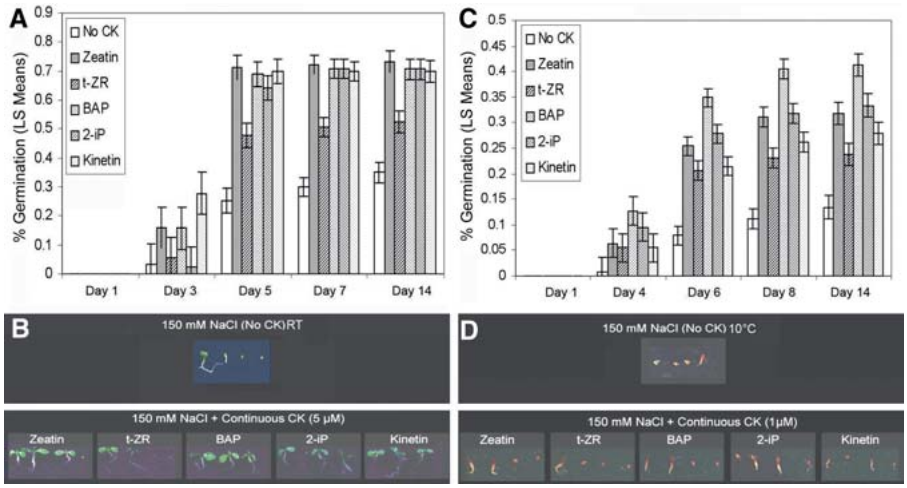

Figure 3. Effects of CK on the wild-type *A. thaliana* when included in the MS medium. (A) Germination and (B) appearance of wild-type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at RT. (C) Germination and (D) appearance of wild-type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at 10°C.

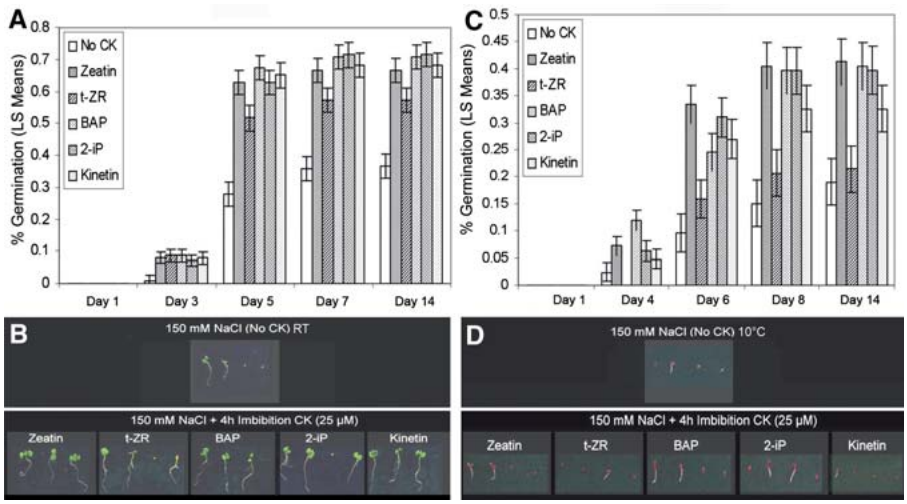

Figure 4. Effects of CKs when wild-type *A. thaliana* seeds were imbibed in aqueous CK solutions. (A) Germination and (B) appearance of wild-type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at RT. (C) Germination and (D) appearance of wild-type *A. thaliana* germinated and grown for 2 weeks in the presence of 150 mM NaCl at 10°C.

Tissue from soil-grown plants was also used for endogenous CK determinations because these plants exhibited differences in the phenotypes (Figure 1) when compared to the wild-type. Some of these differences included precocious flowering, a higher degree of lateral branching, and increased numbers of seed pods (Figure 2), all of which are traits that have been connected to an increase in CK activity (Bonhomme and others 2000; Liu and others 2002; Mader and others 2003; Riefler and others 2006; Tanaka and others 2006). Our results indicated that the total endogenous concentrations of CK in tissues of 2-week-old transgenic lines 6.9 and 25.20 grown on MS plates increased, whereas the total concentrations in tissues from soil-grown plants generally decreased (Table 2). These results provide direct evidence that the expression of pea *ABR17* in *A. thaliana* modulates CK levels.

Our results also indicated that levels of specific forms of CKs were being consistently altered in the transgenic lines. For example, in tissues from plants grown in both conditions, an increase in the concentration of *cis*-Z was observed. Similarly, the endogenous concentration of *cis*-[9RMP]Z also increased in the transgenic lines 6.9 and 25.20 when grown in MS plates, whereas in the soil-grown plants an increase in *cis*-[9RMP]Z was observed only in line 6.9 (Table 2). The endogenous concentration of *trans*-Z, a form of CK that is considered to be biologically active (Yamada and others 2001), also increased in tissue of transgenic lines 6.9 and 25.20 obtained from MS-grown seedlings however, the tissue from soil-grown seedlings did not show any differences in the concentration of this form of CK (Table 2). Another CK that showed consistent increase in both the systems is iP, also considered to be a biologically active form, where the concentrations increased in the two transgenic lines (6.9 and 25.20) when grown on MS plates.

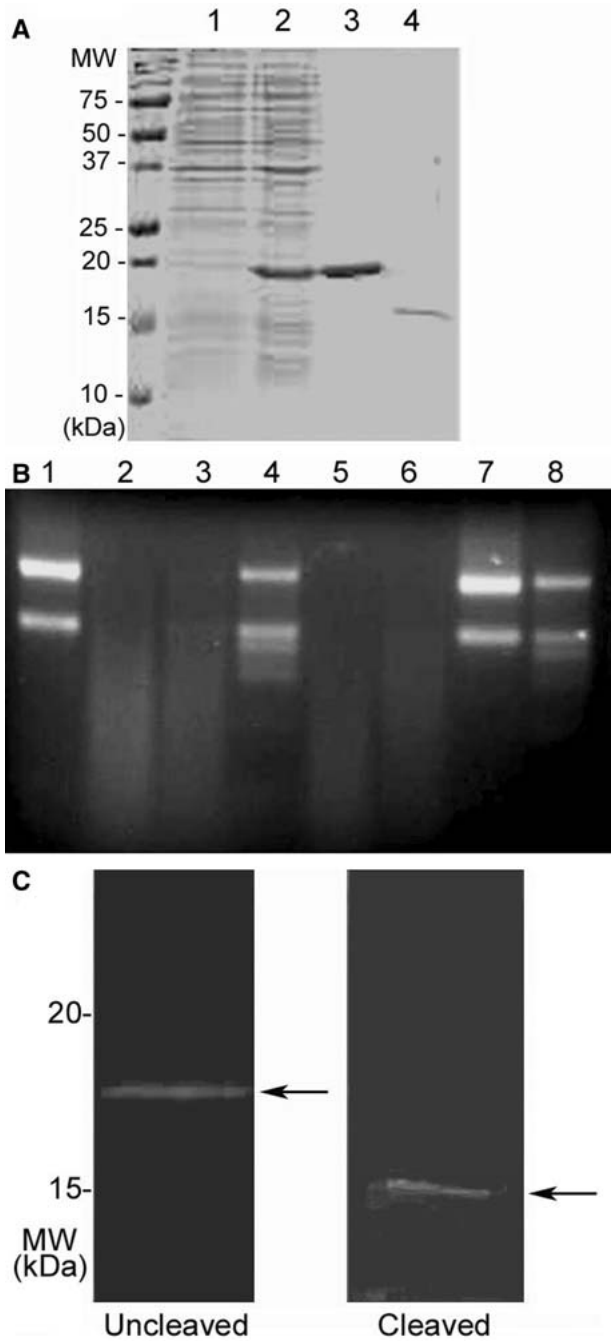

Figure 5. (A) Over expression and purification of recombinant pea ABR17. Lanes 1 and 2 represent uninduced and induced *E. coli* cultures, respectively and lanes 3 and 4 represent purified recombinant ABR17 protein with or without the histidine tag, respectively. (B) In-solution RNA degradation assay with recombinant ABR17. Lanes 1–3 and 7 are reactions with total RNA from pea, and lanes 4–6 and 8 with total RNA from canola. Lanes 2, 5 are reactions with 9 μg recombinant ABR17 protein with the histidine tag and lanes 3, 6 are reactions with 6 μg recombinant ABR17 protein without the histidine tag. Lane 7 is total RNA from pea incubated with 9 μg recombinant ABR17 protein with the histidine tag which had been boiled in a boiling water bath for 15 min and lane 8 is total RNA from canola incubated with recombinant 6 μg ABR17 protein without the histidine tag which had also been boiled for 15 min. (C) In-gel RNase activity. Arrows point to clear bands that are produced as a result of RNase activity and correspond to the molecular weights of recombinant ABR17 with or without the histidine tag.

To correlate the changes in CK profiles with *ABR17* expression, the expression of *ABR17* and the primary CK-responsive gene, *ARR5* (Hwang and Sheen 2001) was investigated in the three transgenic and wild-type lines. Our results indicated that all transgenic lines had greater *ABR17* expression than controls and that this was most pronounced in line 6.9. Lines 14.9 and 25.20 were not significantly different with respect to *ABR17* expression, and this may be due to positional effects of the transgene and/or other uncharacterized differences between these two lines (Table 3). Similarly we observed a fourfold to ninefold increase in the levels of *ARR5* transcript in the transgenic lines, with the highest increase (~9 fold) in line 6.9, which further suggests a direct relationship between *ABR17* and *ARR5* expression in our transgenic *A. thaliana* lines.

The observed differences in the endogenous concentrations of specific forms of CKs in the transgenic lines correlated well with the ability of certain CKs to enhance the germination of wild-type *A. thaliana*. For example, zeatin (mixed isomers) supplied continuously for 2 weeks in the growth media resulted in enhanced germination in the presence of NaCl, as well as under low temperature stress (Figure 3D), which correlated well with increase in the endogenous concentration of both *cis*-Z and *trans*-Z in the transgenic lines 6.9 and 25.20. Similarly, the imbibition of seeds in aqueous solutions of iP prior to germination under these stresses resulted in enhanced germination in the presence of both types of abiotic stresses investigated in this study. These results also agreed well with our observation that the endogenous concentration of iP was elevated in the two transgenic lines, 6.9 and 25.20. Interestingly, the endogenous CK concentrations did not show an increase in line 14.9, which also did not exhibit the enhanced germination and stress tolerance observed with the other two lines (6.9 and 25.20; Srivastava and others 2006b). However, what is consistent is the fact that CKs showed an increase in two of the three transgenic lines tested, and this increase correlates with enhanced germination under abiotic stress.

Cytokinins are plant hormones that regulate many developmental and physiological processes in plants, including promotion of cell division, seed germination, chloroplast development, leaf senescence, lateral shoot development, vascular differentiation, nutrient mobilization, and regulation of gene expression (Mok and Mok 2001; Haberer and Kieber 2002). Cytokinins are known to contribute to release of dormancy, thereby enhancing germination by enhancing ethylene biosynthesis (Leubner-Metzger 2006). This observation is further supported by Chiwocha and others (2005), who observed that in the ethylene-insensitive mutant *etr* 1-2 decreased germination rates correlated well with an increase in CK-conjugation and a decrease in active CKs. In the present study, we observed that both the percentage of seed germination and early seedling development were enhanced in wild-type *A. thaliana* through the exogenous application of various CKs, suggesting that both dormancy release and seedling growth may be promoted by CKs. The increase in endogenous CKs in *ABR17* transgenic seedlings indicates that, in those lines, the increased germination and seedling growth may be mediated through an increase in CKs.

A small number of PR 10 proteins including the pea PR 10.1 have been demonstrated to possess RNase activity; however, the role of RNase activity in their *in planta* function is not clear (Srivastava and others 2006a). In this study we have also demonstrated the RNase activity of pea *ABR17* for the first time and, taken together with an increase in endogenous CK in the *ABR17*-transgenic *A. thaliana* lines, our results suggest the possibility that the RNase activity of *ABR17* may be responsible for the increase in endogenous CKs. Cytokinin biosynthesis in plants may occur via two independent pathways: one, the mevalonate (MVA) pathway, located in the cytosol, which prenylates tRNA and is mainly responsible for producing *cis*-Z derivatives, and a second, localized in plastids, which involves the methylerythritol phosphate (MEP) pathway in providing the prenyl precursor to *trans*-CK and iP-type CK (Kasahara and others 2004; Sakakibara 2006). tRNAs have been suggested to be a possible source of endogenous CKs because of isopentenylation of an adenine residue in these molecules (Swaminathan and others 1977), and it has been estimated that in plants tRNAs may contribute as much as 40%–50% of cytokinin pools (Barnes and others 1980; Letham and Palni 1983; Prinsen and others 1997). It is also suggested that tRNA degradation is a source of *cis*-Z type CKs (Sakakibara 2006), and the presence of iP in tRNA has been reported, albeit as a minor component of plant tRNA (Prinsen and others 1997). Based on the results of Kasahara and others (2004), an increase in the levels of *cis*[9R-MP]Z, *cis*-Z and iP would be predicted in the event of increased tRNA degradation, and this is, in fact, what is being observed in two of the three transgenic lines (6.9 and 25.20). However, the contribution of *de novo* pathways in generating the observed CK profiles cannot be ruled out.

In this study we demonstrate that the constitutive expression of pea *ABR17* cDNA results in an increase in endogenous concentrations of CKs. This increase in endogenous CKs may play a crucial role in enhancing the germination of *A. thaliana* under abiotic stress conditions. This suggestion is further supported by the increased germination of wild-type *A. thaliana* through an exogenous application of CKs. The RNase activity of pea *ABR17* may be involved in enhancing the endogenous CK concentrations; however, the involvement of tRNA independent pathways cannot be ruled out. Additional experiments to test the involvement of RNase activity of *ABR17* in enhancing endogenous CKs are necessary and may include the engineering of *ABR17* to abolish its RNase activity. Additionally, the presence of iP and *cis*-Z in *A. thaliana*, as well as pea tRNA molecules, must be demonstrated together with *ABR17*-mediated release of these CK moieties from the tRNA. Such experiments are in progress in our laboratory and will form the basis of future communications.

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REFERENCES

Barnes MF, Tien ChL , Gray JS. 1980. Biosynthesis of cytokinins by potato cell cultures. Phytochemistry 19:409–412.

Bonhomme F, Kurz B, Melzer S, Bernier G, Jacqmard A. 2000. Cytokinin and gibberellin activate SaMADS A, a gene apparently involved in regulation of the floral transition in *Sinapis alba*. Plant J 24:103–111.

Chiwocha DS, Cutler AJ, Abrams SR, Ambrose SJ, Yang J, and others. 2005. The *etr1-2* mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. Plant J 42:35–48.

Emery RJN, Leport L, Barton JE, Turner NC, Atkins CA. 1998. cis-Isomers of cytokinins predominate in *Cicer arietinum* seeds throughout their development. Plant Physiol 117:1515–1523.

Emery RJN, Ma Qifu , Atkins CA. 2000. The forms and sources of cytokinins in developing *Lupinus albus* seeds and fruits. Plant Physiol 123:1593–1605.

Ferguson BJ, Wiebe EM, Emery RJN, Guinel FC. 2005. Cytokinin accumulation and an altered ethylene response mediate the pleiotropic phenotype of the pea nodulation mutant R50 (sym16). Can J Bot 83:989–1000.

Fujimoto Y, Nagata R, Fukasawa H, Yano K, Azuma M, and others. 1998. Purification and cDNA cloning of cytokinin-specific binding protein from mung bean (*Vigna radiata*). Eur J Biochem 258:794–802.

Haberer G, Kieber JJ. 2002. Cytokinins. New insights into a classic phytohormone. Plant Physiol 128:354–362.

Hwang I, Sheen J. 2001. Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature 413:383–389.

Iturriaga EA, Leech MJ, Barratt DHP, Wang TL. 1994. Two ABA-responsive proteins from pea (*Pisum sativum* L.) are closely related to intracellular pathogenesis-related proteins. Plant Mol Biol 24:235–240.

Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, and others. 2004. Distinct isoprenoid origins of cis- and trans-zeatin biosyntheses in *Arabidopsis*. J Biol Chem 279:14049–14054.

Kav NNV, Srivastava S, Goonewardene L, Blade SF. 2004. Proteome-level changes in the roots of *Pisum sativum* L. in response to salinity. Ann Appl Biol 145:217–230.

Letham DS, Palni LMS. 1983. The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol 34:163–197.

Leubner-Metzger G. 2006. Hormonal interactions during seed dormancy release and germination In: Amarjit SB editor. *Handbook of Seed Science.* Binghamton, NY, USA: Haworth's Food Products Press. pp 303–341.

Liu X, Huang B, Banowetzb G. 2002. Cytokinin effects on creeping bentgrass responses to heat stress I. shoot and root growth. Crop Sci 42:457–465.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-$\Delta^{\Delta Ct}$ method. Methods 25:402–408.

Mader JC, Turnbull CGN, Emery RJN. 2003. Transport and metabolism of xylem cytokinins during lateral bud release in decapitated chickpea (*Cicer arietinum* L.) seedlings. Physiol Plant 117:118–129.

Markovic-Housely Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, and others. 2003. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J Mol Biol 325:123–133.

Mogensen JE, Wimmer R, Larsen JN, Spangfort MD. 2002. The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. J Biol Chem 277:684–692.

Moiseyev GP, Beintema JJ, Fedoreyeva LI, Yakovlev GI. 1994. High sequence similarity between a ribonuclease from ginsgeng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta 193:470–472.

Mok DW, Mok MC. 2001. Cytokinin metabolism and action. Annu Rev Plant Physiol Plant Mol Biol 52:89–118.

Pasternak O, Biesiadka J, Dolot R, Handschuh L, Bujacz G, and others. 2005. Structure of a yellow lupin pathogenesis-related PR10 protein belonging to a novel subclass. Acta Crystallogr D Biol Crystallogr 61:99–107.

Prinsen E, Kaminek M, van Onckelen HA. 1997. Cytokinin biosynthesis: a black box? Plant Growth Regul 23:3–15.

Riefler M, Novak O, Strnad M, Schmulling T. 2006. *Arabidopsis* cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 18:40–54.

Sakakibara H. Cytokinins: activity, biosynthesis, and translocation. 2006. Annu Rev Plant Biol 57:431–449.

Sikorski MM, Biesiadka J, Kasperska AE, Kopcinska J, Lotocka B, and others. 1999. Expression of genes encoding PR10 class pathogenesis-related proteins is inhibited in yellow lupine root nodules. Plant Sci 149:125–137.

Srivastava S, Fristensky B, Kav NNV. 2004. Constitutive expression of a PR 10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol 45:1320–1324.

Srivastava S, Emery RJN, Kurepin LV, Reid DM, Fristensky B, Kav NNV. 2006a. Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels. Plant Growth Regul 49:17–25.

Srivastava S, Rahman MH, Shah S, Kav NNV. 2006b. Constitutive expression of the pea ABA-responsive (*ABR17*) cDNA confers multiple stress tolerance in *Arabidopsis thaliana*. Plant Biotech J 4:529–549.

Swaminathan S, Bock R, Skoog F. 1977. Subcellular localization of cytokinins in transfer ribonucleic acid. Plant Physiol 59:558–563.

Tanaka M, Takei K, Kojima M, Sakakibara H, Mori H. 2006. Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. Plant J 45:1028–1036.

Tewari S, Brown SM, Kenyon P, Balcerzak M, Fristensky B. 2003. Plant defense multigene families. II. Evolution of coding sequence and differential expression of PR10 genes in *Pisum.* http://arXiv.org/q-bio.PE/0310038.

van Loon LC, van Strien EA. 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55:85–97.

Warner SAJ, Gill A, Draper J. 1994. The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. Plant J 6:31–43.

Wu F, Li YK, Chang S, Zhou Z, Wang F, and others. 2002. Purification, characterization and preliminary crystallographic studies of a PR 10 protein from *Pachyrrhizus erosus* seeds. Acta Crystal D 58:2165–2167.

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, and others. 2001. The Arabidopsis AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol 42:1017–1023.