Plant Physiol. (1997) 114: 1177–1185

ZEA3: A Negative Modulator of Cytokinin Responses in Plant Seedlings[1]

Thomas Martin[2], Bruno Sotta, Marc Jullien, Michel Caboche, and Jean-Denis Faure[3]*

Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, route de St. Cyr, 78026 Versailles cedex, France (T.M., M.J., M.C., J.-D.F.); and Laboratoire de Physiologie du Developement des Plantes, Unité de Recherche Associée 1180 Centre National de la Recherche Scientifique, Universite Pierre et Marie Curie F-75252 Paris cedex 05, France (B.S.)

In *Nicotiana plumbaginifolia* cytokinins affect seedling development by inhibiting root growth and hypocotyl elongation and by stimulating cotyledon expansion. The *zea*3.1 mutant was selected for its inability to grow in conditions of low nitrogen and for its ability to grow independently on inhibitory concentrations of zeatin (J.D. Faure, M. Jullien, M. Caboche [1994] Plant J 5: 481–491). The zea3.1 growth response to cytokinins is reflected by an increase in cotyledon expansion due to cell division and by a swelling of the hypocotyl due to cell enlargement. An analysis of the seedling's root length and fresh weight over a wide range of benzyladenine concentrations showed that *zea*3.1 plants exhibit a higher sensitivity and an amplified response to cytokinins. A similar response of *zea*3.1 to benzyladenine was also seen in the expression of *msr*1, a cytokinin-regulated gene. Regulation of *msr*1 expression by protein phosphorylation was unaffected by the *zea*3.1 mutation. No significant differences in cytokinin and auxin levels were found between *zea*3.1 and wild-type seedlings, suggesting that the mutant phenotype is not caused by an alteration of these hormone levels. The data presented suggest that ZEA3 negatively modulates cytokinin responses and may function as a broad regulator of seedling development.

Cytokinins were originally identified by their role in plant organogenesis and by their ability to regulate the division of plant cells in combination with auxin (for review, see Krikorian [1995]). Today it is known that cytokinins are involved in many aspects of plant growth and development, such as apical dominance, photomorphogenesis, chloroplast biogenesis and maintenance, assimilate mobilization and translocation, and senescence (for reviews, see Binns [1994]; Mok [1994]). Unfortunately, plant responses to exogenously supplied cytokinins are slow and influenced by environmental factors or other hormones. The complex nature of responses to cytokinins have hindered the biochemical and molecular analyses of cytokinin action. Therefore, despite the involvement of cytokinins in a large number of processes, their mode of action remains almost unknown (Davis, 1995).

As for other hormone systems in animals and plants, it can be assumed that cytokinins first bind to a receptor protein, inducing a cascade of reactions that finally mediate the expression of target genes. In fact, several cytokinin-binding proteins have been purified, but their confirmation as cytokinin receptors awaits the development of suitable test systems (Binns, 1994). Similarly, several cytokinin-regulated genes have also been isolated (for review, see Binns [1994]). Most of these are also regulated by other hormones, light, nutritional status, or various stresses. One of the best-characterized examples of kinetics and sensitivity of the response is the *msr*1 (*m*ultiple *s*timulus *r*esponse) gene from *Nicotiana plumbaginifolia* (Dominov et al., 1992). This gene is a member of a family of environmentally responsive genes that are related to the stringent-starvation response from *Escherichia coli*. The expression of *msr*1 is transiently induced in the *N. plumbaginifolia* cell culture by auxin and cytokinin, although the kinetics differ between the two hormones. The cytokinin response peaks 24 h after hormone application, whereas the auxin response peaks earlier (3–6 h). It is interesting that the cytokinin pretreatment enhanced a subsequent auxin treatment. Dominov and co-workers proposed that cytokinin may sensitize an auxin response or block a feedback inhibition of the auxin response.

The genetic analysis of hormone mutants has been used successfully to investigate the mode of action of various plant hormones. To analyze the cytokinin response in higher plants, mutants have been isolated by selecting phenotypes similar to cytokinin-overproducing or -insensitive plants (Wang, 1994). Two *Arabidopsis thaliana* mutants belonging to the first category have been described: *ampl* (*a*ltered *m*eristem *p*rogram), which has an increased level of endogenous cytokinin, and *det1* (*de-et*iolated), which seems to have a higher sensitivity to cytokinins (Chory et al., 1991, 1994; Chaudhury et al., 1993). Several Arabidopsis-insensitive mutants, *cyr1* (*cy*tokinin *r*esponse), *stp1* (*st*unted *p*lant), and *ckr1* (*cy*tokinin *r*esistance), were isolated on the basis of root elongation on inhibitory concentrations of cytokinin (Su and Howell, 1992; Baskin et al.,

[1] This work was supported by European Economic Community project no. BIO2-CT93-0400.

[2] Present address: Plant Molecular Sciences Group, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, UK.

[3] Present address: Boyce Thompson Institute, Cornell University, Ithaca, NY 14853–1801.

* Corresponding author; e-mail jdf14@cornell.edu; fax 1–607–254–1242.

Abbreviations: CPPU, 1-(6-chloropyrid-4-yl)-3-phenylurea; PU-14, 1-phenyl-3-(tetrazolo[1,5-a]pyrid-7-yl)urea.

1995; Deikman and Ulrich, 1995). Whereas *cyr1* and *stp1* seem to be specific to cytokinin, *ckr1* was shown to be insensitive to ethylene and allelic to the ethylene-insensitive mutant *ein2* (Cary et al., 1995). In *N. plumbaginifolia* the *ckr1/iba/esg* mutant was classified as cytokinin-resistant because it germinates on higher cytokinin concentrations than the wild type, and the mutant leaves continue to develop at cytokinin concentrations inhibitory to the wild type (Bitoun et al., 1990; Blonstein et al., 1991; Parry et al., 1991). As with the Arabidopsis *ckr1* mutant, the *ckr1/iba/esg* mutation is not cytokinin-specific. This mutant has a defect in the last step of ABA biosynthesis and is also insensitive to auxin and to the GA_3 biosynthesis inhibitor paclobutrazol (Bitoun et al., 1990; Blonstein et al., 1991; Parry et al., 1991).

The *zea* mutants were isolated for their cotyledon expansion induced by a high zeatin concentration (Jullien et al., 1992) and classified into three complementation groups called *Zea*1 (three mutants), *Zea*2 (two mutants), and *Zea*3 (mutant 203.1). All *zea* mutations were monogenic and recessive. On cytokinin-supplemented media, *zea* seedlings developed cotyledon and hypocotyl hypertrophy, whereas wild-type seedling growth was inhibited. In the absence of cytokinins the *zea*3 mutant exhibited a specific developmental phenotype. The mutant germinates with fused cotyledons that never separate (Faure et al., 1994). A new mutant presenting a similar fused cotyledon phenotype (mutant 203.6) was identified in a screen for nitrate reductase regulatory mutants on the basis of its inability to grow on low-nitrogen medium (Faure et al., 1994). This growth defect was due to the low nitrogen to Suc ratio in the culture medium and could be reversed by increasing the concentration of nitrogen in relation to Suc. Genetic analysis showed that 203.1 and 203.6 were allelic and probably identical (Faure et al., 1994). Thus, the alteration of cotyledon development, primary metabolism, and cytokinin-growth responses appeared to be linked to a single locus, *Zea3*.

To understand the role of *zea3* in cytokinin responses, we undertook a detailed analysis of the developmental and the molecular responses of *zea*3.1 to cytokinins. The results show that the development of seedlings and the expression of a cytokinin-activated gene are hypersensitive to cytokinins in *zea*3.1. *zea*3.1 displayed a normal level of cytokinins, suggesting that the mutation alters the hormone sensitivity. The ZEA3 protein would negatively regulate cytokinin responses during the germination of plant seedlings.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of the haplo-diploid *Nicotiana plumbaginifolia* cv Viviana line pbHID and the *zea*3.1 mutant (Faure et al., 1994) were germinated on B medium (Caboche, 1987) supplemented with 10 mM nitrate (control conditions). Seed dormancy was broken by keeping the cultures for the first 10 d under low-temperature/low-light conditions before transferring them to standard culture conditions for 10 d more (Faure et al., 1994). Seedlings were harvested at 3 weeks, when the cotyledons were fully expanded but before the first leaves appeared.

Hormone Treatments

BA was purchased from Sigma, and CPPU and PU-14 were kindly provided by Michel Laloue (Institut National de le Recherche Agronomique, Versailles, France). BA, CPPU, and PU-14 were dissolved in DMSO. The final DMSO concentration never exceeded 1% (v/v). For short-term treatments, the substrates were diluted in 3 mL of liquid B medium on the top of plates. Control seedlings were treated with 3 mL of liquid B medium and the appropriate DMSO concentration.

Root Length and Fresh Weight Measurements

The fresh weight is given as the average fresh weight per seedling calculated from three pools of 10 seedlings.

The root length was measured by harvesting 30 seedlings, aligning and drying them on plastic covers at room temperature, and scanning the roots using the VISILOG software, as described by Orbovic and Kieu (1996).

RNA Analysis

Seedlings were harvested and frozen in liquid nitrogen. Total RNA was extracted and analyzed as described previously (Kay et al., 1987; Sambrook et al., 1989). Hybridization using nylon membranes (Hybond N^+, Amersham) was performed in the presence of 50% formamide and 5× SSPE at 42°C using randomly [^{32}P]dCTP-labeled probes. The membranes were washed at 65°C in 0.2× SSPE and 0.5% SDS and subsequently autoradiographed; they were rehybridized several times. Between each hybridization the membranes were stripped for 2 h at 65°C in 0.1% SDS, 0.001 M EDTA, and 0.005 M Tris, pH 7.5. A 0.9-kb *Xho*I/*Bam*HI cDNA insert of pLS216 (Dominov et al., 1992) was used to analyze *msr*1 expression. The β-ATP synthase probe (ATPase) was the complete cDNA from the β-subunit of *N. plumbaginifolia* (Boutry and Chua, 1985). The relative RNA accumulation was analyzed in all of the experiments as described below.

Computer Analysis of RNA Expression

An XRS (3 CX:6 CS) scanner attached to a Sun Sparc IPC microcomputer with a color monitor (Bioimage, Ann Arbor, MI) was used as hardware. Bands were quantified using one-dimensional analysis software (Visage, version 4.6, Bioimage). Each autoradiograph was scanned as described previously (Santoni et al., 1994). The relative RNA accumulation was calculated by dividing the relative *msr*1 expression by the relative expression of the β-ATPase. The relative expression was calculated by dividing each spot-quantified value by the highest value of the autoradiograph. For clarity, the highest relative RNA accumulation of the wild type was set at 100% in each experiment.

Light Microscopy

Seedlings were vacuum-infiltrated with 0.2% glutaraldehyde and 4% formaldehyde and subsequently fixed for 24 h at 4°C. Following a dehydration in ethanol the seedlings were embedded in Historesin TM (Leica). Sections (3–5 μM) were made with a microtome (Jung RM 2055, Leica) and stained with toluidine or methylene blue.

Hormone Measurements

Auxin and cytokinin concentrations were analyzed by ELISA, as previously described (Kraepiel et al., 1995). The material used in the experiments was 3-week-old seedlings grown in a controlled culture chamber. Several hundred seedlings were collected at a time, frozen in liquid nitrogen, and lyophilized prior to grinding into powder. Extractions were performed in nonoxidative methanol:water (80:20, v/v) followed by a prepurification step and fractionation with HPLC (Beckman) in a 0.2% formic acid:methanol gradient. The fractions collected were analyzed by ELISA with polyclonal antibodies against IAA (Julliard et al., 1992), zeatin, and isopentenyl adenosine (Kraepiel et al., 1995). The dosages were determined at least five times on independent batches of seedlings.

RESULTS

BA Induces Cotyledon and Hypocotyl Hypertrophy in Wild-Type and *zea*3.1 Seedlings

The phenotypes of *zea*3.1 and wild-type seedlings grown for 3 weeks in the presence of 0 to 50 μM BA are shown in Figure 1. In the absence of BA the mutant had fused cotyledons, longer roots, and a shorter hypocotyl than the wild type (Fig. 1, A and C). A variety of phenotypic changes was observed with increasing BA concentration, including inhibition of hypocotyl elongation in the wild type and inhibition of root growth and swelling of the hypocotyl in both the wild type and the mutant (Fig. 1, A–C). Swelling of the hypocotyl and expansion of the cotyledons were exaggerated in *zea*3.1 at all BA concentrations tested (Fig. 1, A and C). The response of the wild-type cotyledons to BA was more complex and concentration-dependent. At low concentrations (1–100 nM BA) cotyledon expansion was inhibited, whereas at higher concentrations (1–10 μM BA) the inhibition was partially reversed (Fig. 1A). Further increase in the BA concentration to 50 μM completely blocked the development of wild-type seedlings. The mutant cotyledons and hypocotyls were still able to expand at 50 μM BA and in some cases adventitious shoots developed on hypocotyls after 3 weeks of growth (Fig. 1A; data not shown).

BA-Induced Hypertrophies Are Associated with Cell Division and Cell Enlargement

In the absence of BA the mutant hypocotyl is much shorter than the wild-type hypocotyl. Longitudinal hypocotyl sections failed to show any differences in cell size or shape between the wild type and the mutant (Fig. 2, A and E), suggesting that the shorter hypocotyl of *zea*3.1 was caused by a reduced number of cells along the hypocotyl. The meristem of *zea*3.1 seedlings showed no obvious differences in cell size or cell number compared with the wild type (Fig. 2, A and E). The overall cotyledon structure was similar in both the wild type and mutant, except that the cells were larger in the mutant and the cotyledons were fused along their epidermis (Fig. 2, C and E; Faure et al. [1994]).

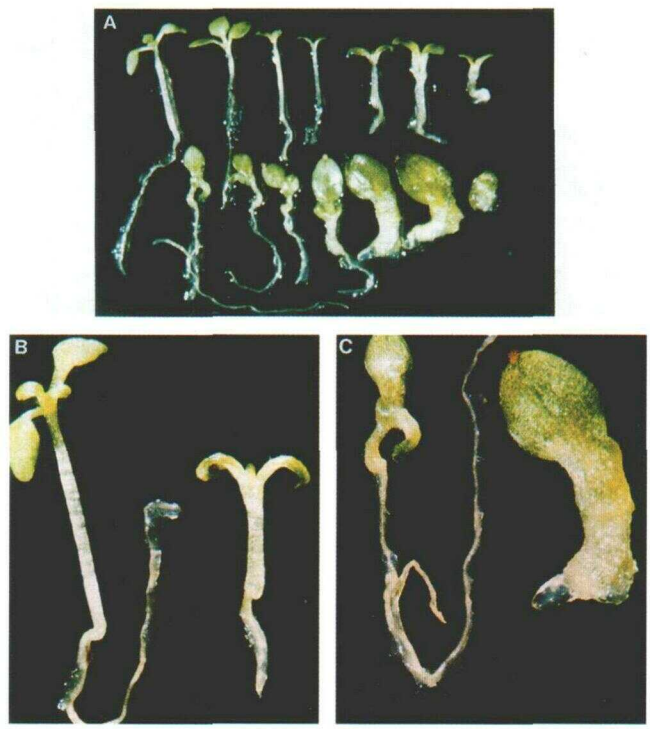

Figure 1. Phenotypes of wild-type and *zea*3.1 seedlings. A, Comparison of wild-type and *zea*3.1 morphologies on increasing cytokinin concentrations. Seedlings of wild-type (top) and *zea*3.1 (bottom) were grown for 21 d in the presence of 0, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 50 μM BA (left to right). B, Wild-type phenotype in the presence of cytokinin. Wild-type seedlings were grown for 21 d in the absence (left) or in the presence (right) of 5 μM BA. C, Phenotype of *zea*3.1 in the presence of cytokinin. The *zea*3.1 seedlings were grown for 21 d in the absence (left) or in the presence (right) of 5 μM BA.

In the presence of BA *zea*3.1 and, to a lesser extent, the wild type showed cell enlargement of the hypocotyl and cell proliferation in the cell layers below the meristem (Fig. 2, B and F). The BA-induced response in the hypocotyl of *zea*3.1 seedlings was more pronounced (compare B and F in Fig. 2). BA promoted greater cell proliferation in cell layers below the meristem in *zea*3.1 (Fig. 2, B and F). Cotyledons were enlarged in both the wild type and mutant in response to BA. In the wild type this enlargement was due to an increase of the cell size, whereas the number of cells and cell layers did not change (Fig. 2, C and D). In the mutant the enlargement was mainly due to an increase in cell numbers, whereas cell size did not change significantly (Fig. 2, E and F).

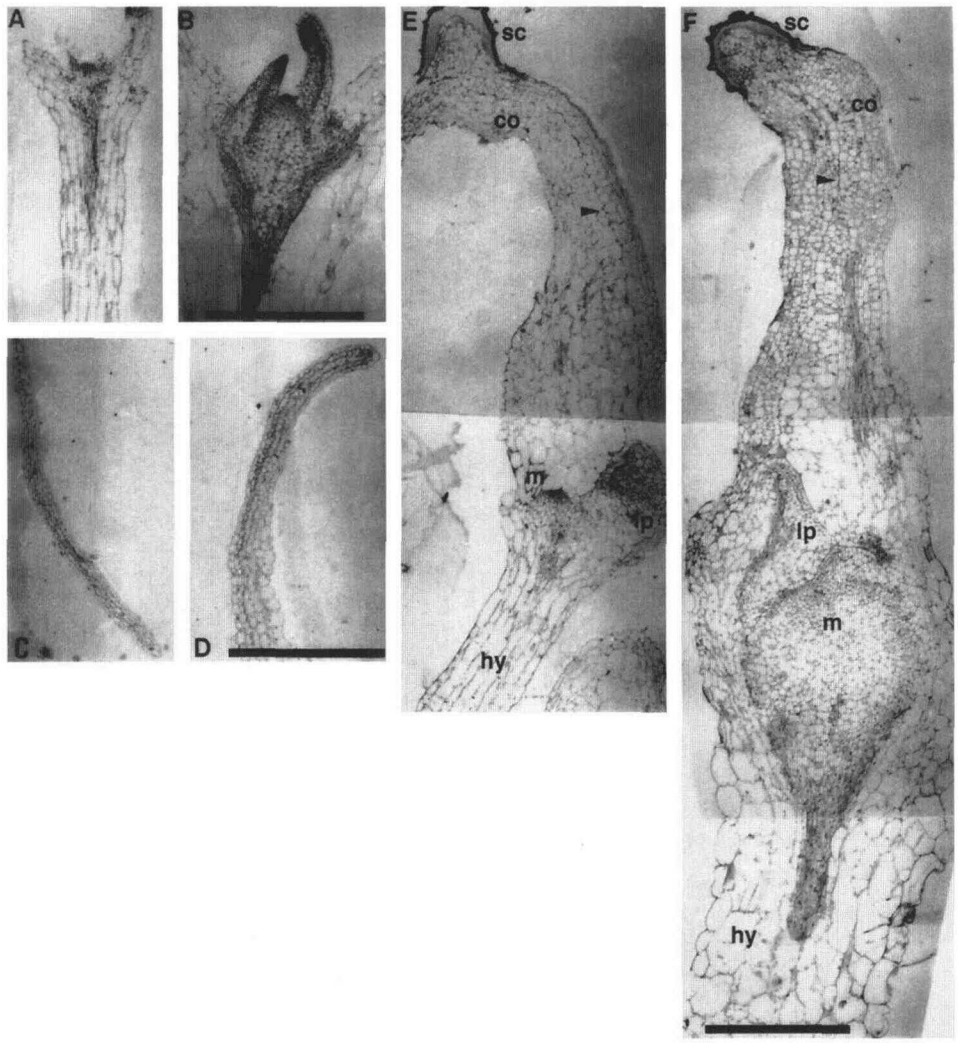

Figure 2. Sections of wild-type and *zea*3.1 seedlings. A and B, Longitudinal sections of wild-type hypocotyls in the absence (A) and in the presence (B) of 5 μM BA. C and D, Longitudinal sections of wild-type cotyledons in the absence (C) and in the presence (D) of 5 μM BA. E and F, Longitudinal sections of *zea*3.1 seedlings in the absence (E) and in the presence (F) of 5 μM BA. The black arrowheads show the fusion zone of the cotyledons. m, Meristem; co, cotyledon; sc, seed coat; lp, leaf primordia; and hy, hypocotyl. All sections are at the same magnification and the bars represent 800 μm.

*zea*3.1 Has Greater Sensitivity to BA

Root length and fresh weight were analyzed in wild-type and mutant seedlings grown on a wide range of BA concentrations, as shown in Figure 3. In the absence of BA, the roots of the *zea*3.1 seedlings were twice as long as those of the wild-type (Fig. 3A). Increasing concentrations of BA inhibited wild-type and *zea*3.1 root growth, but the degree of inhibition was greater for *zea*3.1 and occurred at lower concentrations of BA than for the wild type (Fig. 3A). Fifty-percent inhibition of root growth was observed at 10 nM for *zea*3.1 and at 50 nM for the wild type. Mutant root growth also showed greater inhibition by BA. Both the wild type and the mutant exhibited maximum inhibition at 1 μM BA, but maximum root inhibition was 95% for mutant roots compared to 60% for wild-type roots.

Measurements of seedling fresh weight, as shown in Figure 3B, were used to examine the effects of BA on cotyledon and hypocotyl growth. The normalized fresh weight of mutant seedlings grown in the absence of BA was 35% lower than the fresh weight of wild-type seedlings germinated under the same conditions. In wild-type seedlings low BA concentrations (0.001–0.01 μM) reduced fresh weight to 30% of seedlings grown without BA (Fig. 3B). At higher BA concentrations (1–10 μM), expansion of the cotyledons accounted for increased wild-type fresh weight to 65% of those grown without BA (Fig. 3B). In contrast to the wild type, the fresh weight of the *zea*3.1 seedlings never decreased, but slightly increased, at low BA concentrations (Fig. 3B). Because of cotyledon hypertrophy, the *zea*3.1 fresh weight further increased with increasing BA concentrations and reached a maximum at 1 μM BA, 4 times higher than the fresh weight of seedlings grown without BA (Fig. 3B).

Comparison of the relative BA dose response of fresh weight increase and root growth inhibition showed that in

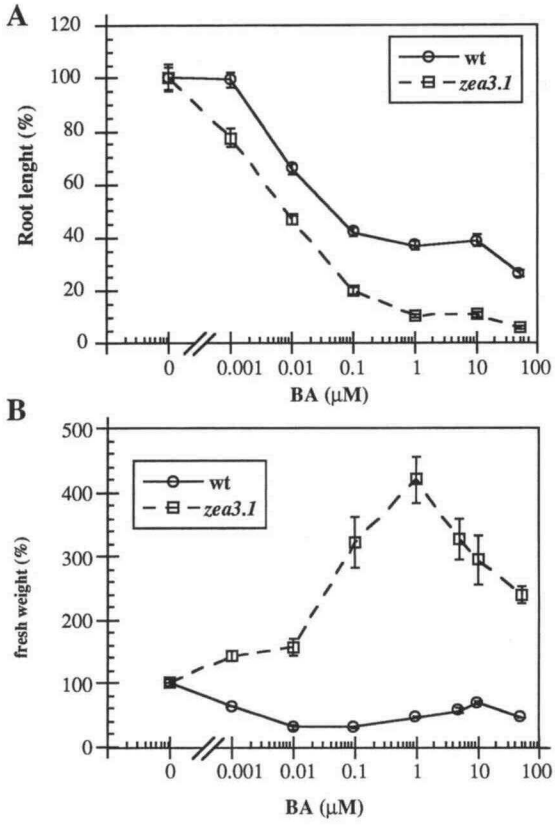

Figure 3. A, Cytokinin-induced root-length inhibition in wild-type and *zea*3.1 seedlings. The root lengths of 3-week-old wild-type and *zea*3.1 seedlings germinated in the presence of increasing BA concentrations were measured. Values are the means of 30 seedlings and are expressed (mean and SE) as percentages of control root length (without BA). The root lengths of the wild type and *zea*3.1 in the absence of BA are 6.14 ± 0.32 and 11.07 ± 0.43 mm, respectively. B, Cytokinin-induced fresh weight variation in wild-type and *zea*3.1 seedlings. Fresh weights of 3-week-old wild-type (wt) and *zea*3.1 seedlings germinated in the presence of increasing BA concentrations were measured. Values correspond to the means of three replicates of 10 seedlings and are expressed (mean and SE) as percentages of control fresh weight (without BA). The fresh weights of the wild type and *zea*3.1 in the absence of BA are 2.72 ± 0.12 and 1.78 ± 0.18 mg per seedling, respectively.

*zea*3.1 responses were shifted to lower cytokinin concentrations. Additionally, the amplitudes of these responses in both cases were higher in the mutant than in the wild type. This indicates that *zea*3.1 is more sensitive and has an amplified response to BA.

The Expression of *msr*1, a Cytokinin-Regulated Gene, Is Regulated but Overexpressed in *zea*

Morphological responses in *zea*3.1 were more sensitive to BA than in the wild type. To determine whether cytokinin-induced gene expression was also more sensitive, the expression of the cytokinin- and auxin-regulated *N. plumbaginifolia* gene *msr*1 was analyzed (Dominov et al., 1992). Figure 4A shows *msr*1 mRNA expression in mutant and wild-type seedlings grown for 3 weeks in the absence or

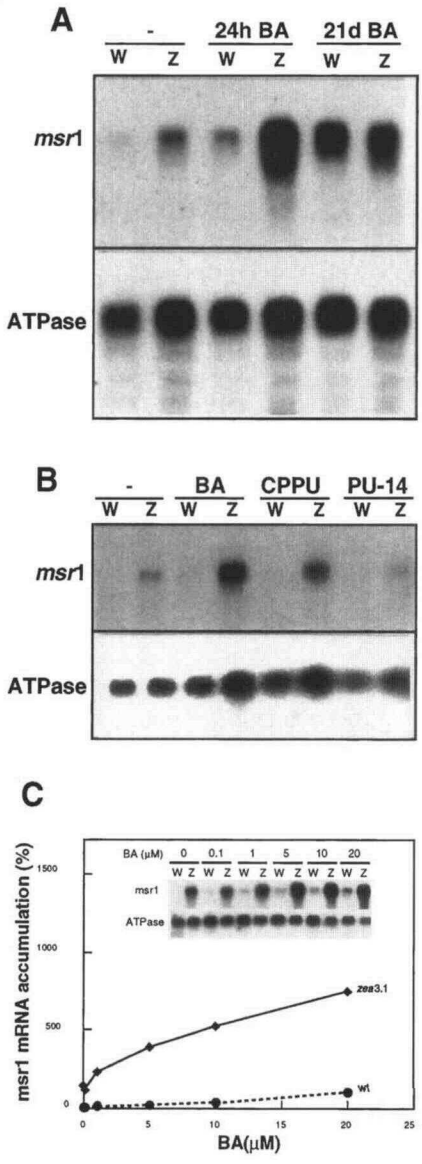

Figure 4. Regulation of *msr*1 expression in wild-type and *zea*3.1 seedlings by cytokinins. A, Comparison of BA induction of *msr*1 in wild-type and *zea*3.1 seedlings. Wild-type (W) and *zea*3.1 (Z) seedlings were germinated for 21 d on B medium (−), B medium for 20 d and supplemented with 5 μM BA for 24 h (24h), or B medium supplemented with 5 μM BA for 21 d (21d). Ten micrograms of total RNA was used per sample for northern-blot analysis to detect the expression of the *msr*1 gene. A β-ATPase-specific probe was used for control hybridization. B, Induction of *msr*1 in wild-type and *zea*3.1 seedlings by active cytokinins. Wild-type (W) and *zea*3.1 (Z) seedlings were germinated for 21 d on B medium (−) or B medium supplemented for 24 h with 5 μM BA (BA), 5 μM CPPU, or 5 μM PU-14. Northern analysis was performed as described in A. C, Effect of BA concentration on *msr*1 expression in wild-type and *zea*3.1 seedlings. Wild-type (W or wt) and *zea*3.1 (Z) seedlings were germinated for 21 d on B medium and supplemented with 0 to 20 μM BA for 24 h. Ten micrograms of total RNA was used per sample for northern-blot analysis using the *msr*1 (inset, top row) and the β-ATPase-specific (bottom row) probes. The relative *msr*1 RNA accumulation values presented here were calculated as described in "Materials and Methods." For clarity, the highest relative RNA accumulation of the wild type was set at 100% (20 μM BA).

presence of 5 μM BA (lanes − and 21d BA). The basal level of *msr*1 expression was nearly 3 times higher in the mutant than in the wild type. Growth on 5 μM BA increased the accumulation of *msr*1 mRNA in wild-type seedlings about 9-fold. Mutant seedlings also showed an increased level of *msr*1 mRNA but at about 3- to 4-fold. Consequently, the expression levels in wild-type and mutant seedlings were similar. It has been reported, however, that *msr*1 expression is inducible in cell culture by a 24-h BA treatment (Dominov et al., 1992). Hence, *msr*1 expression was used to analyze the short-term response of wild-type and *zea*3.1 seedlings to BA. Seedlings were grown for 3 weeks and then supplied with 5 μM BA for 24 h and *msr*1 expression was analyzed (Fig. 4A, lanes 24 h BA). A weaker induction of *msr*1 expression, only 2.5 times, was seen in wild-type seedlings when incubated with BA for 24 h compared with 3 weeks. In *zea*3.1 seedlings *msr1* expression was already 3 times higher in the absence of BA, but a further 4- to 5-fold increase was observed after a 24-h BA treatment. Thus, the expression of *msr*1 following application of BA for 24 h was stronger in *zea*3.1 than in wild-type seedlings. The expression of *msr1* was also induced by a urea-type cytokinin, CPPU, but not by an inactive cytokinin derivative, PU-14 (Dias et al., 1995) (Fig. 4B). The induction by 5 μM CPPU, however, was less pronounced than by the same concentration of BA.

Analysis of the *msr*1 mRNA level in *zea*3.1 and the wild type revealed an increase in the short-term response to BA in the mutant. To compare the relative sensitivity of *zea*3.1 and the wild type to BA, the expression of *msr*1 was monitored over a wide range of BA concentrations (Fig. 4C). As expected, both the wild type and *zea*3.1 showed an increase in *msr1* expression with increasing BA concentrations. However, the increase in *msr*1 expression was significantly higher in *zea*3.1 seedlings than in the wild type (Fig. 4C), indicating that *zea*3.1 is more sensitive to BA regarding the expression of *msr*1.

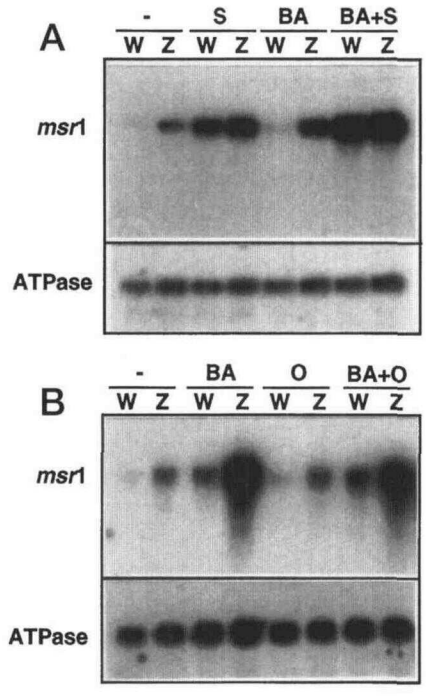

Figure 5. Influence of BA, staurosporin, and okadaic acid on *msr*1 expression. A, BA-independent induction of *msr*1 expression by staurosporin. Wild-type (W) and *zea*3.1 (Z) seedlings were germinated for 3 weeks on B medium (−) and supplemented with 10 μM staurosporin (S), 5 μM BA (BA), or the two in combination (BA+S). Seedlings were harvested 24 h after the beginning of the treatment. B, Okadaic acid did not change the expression of *msr*1 in the wild type and *zea*3.1. Wild-type (W) and *zea*3.1 (Z) seedlings were germinated for 3 weeks on B medium (−) and supplemented with 5 μM BA for 24 h (BA), 0.2 μM okadaic acid for 36 h (O), or 0.2 μM okadaic acid for 36 h and 5 μM BA for 24 h (BA+O). Seedlings were harvested 36 h after the beginning of the okadaic acid treatment.

Protein Phosphorylation Regulates *msr*1 Expression Independently of ZEA3

The hormone induction of *msr*1 expression is affected by protein kinases and phosphatases (Dominov et al., 1992). We examined the effects of kinase and phosphatase inhibitors on the hormone induction of *msr*1 in the *zea*3.1 mutant to determine whether ZEA3 function is related to protein phosphorylation. Staurosporin (a protein kinase inhibitor) and okadaic acid (a protein phosphatase 1 and 2A inhibitor) (Haystead et al., 1989) were used to analyze the influence of protein phosphorylation on *msr*1 expression. The incubation of 3-week-old seedlings with 10 μM staurosporin for 24 h stimulated *msr*1 expression in the wild type and in the mutant (Fig. 5A) and resulted in a similar level of expression in both. This suggested that *msr*1 expression is under negative control by the action of a protein kinase and that this control is still operative in the mutant. The combined treatment of 5 μM BA and 10 μM staurosporin for 24 h caused a similar stimulation of *msr*1 expression in wild-type and *zea*3.1 seedlings (Fig. 5A). However, *msr*1 expression was increased in comparison with the application of staurosporin alone, suggesting that the effects of BA and staurosporin are additive. The treatment of 3-week-old seedlings with 0.1 μM okadaic acid for 36 h did not affect significantly *msr*1 expression in wild-type and *zea*3.1 seedlings (Fig. 5B). Similarly, a pretreatment with 0.1 μM okadaic acid for 12 h before the application of 5 μM BA for 24 h did not change the *msr*1 expression level in either type of seedling compared with BA alone (Fig. 5B). Okadaic acid-sensitive phosphatases do not seem to regulate *msr*1 expression in seedlings. Conversely, staurosporin-sensitive kinases repress *msr*1 expression.

The *zea*3.1 Mutant Has a Normal Level of Cytokinins and Auxins

Increased sensitivity of the *zea*3.1 mutant to BA at either the growth level or the mRNA level could be accounted for by an increase in the endogenous cytokinin level. Cell proliferation in the *zea*3.1 cotyledons and meristem may also be caused by an imbalance in auxin/cytokinin concentrations. To test whether *zea*3.1 phenotypes were caused by abnormal levels of auxin and cytokinins, we analyzed endogenous concentrations of both types of hormones in

3-week-old whole seedlings. Table I shows that there was no significant difference in isopentenyladenine, isopentenyl adenosine, zeatin riboside, 9-glucopyranosyl-zeatin, or IAA in the mutant compared with the wild type. The zeatin concentration was too low to be detected in our samples.

DISCUSSION

Cytokinins such as BA inhibit root and hypocotyl elongation and stimulate hypocotyl thickening and cotyledon expansion during development of *N. plumbaginifolia* seedlings. Similar but more pronounced responses to BA have been observed in the *zea*3.1 mutant. In the presence of BA, root length was 2-fold shorter and fresh weight increase was 4 times higher in the mutant compared with the wild type. These responses occurred at lower BA concentrations in the mutant than in the wild type, indicating that *zea*3.1 is more sensitive to cytokinins. Increased sensitivity of *zea*3.1 to BA was confirmed at the molecular level with the analysis of *msr*1 expression. In *N. plumbaginifolia* cell cultures *msr*1 mRNA accumulation is stimulated by cytokinin as well as by auxin, but the kinetics, amplitude, and negative feedback regulation of *msr*1 expression by the two hormones are different (Dominov et al., 1992). Analysis of *msr*1 expression in wild-type seedlings revealed that active forms of purine and urea cytokinins stimulated *msr*1 mRNA accumulation, whereas the inactive forms did not. Cytokinin induction is more pronounced in *zea*3.1 and occurs at lower BA concentrations, confirming that the mutant perceives cytokinin with higher sensitivity. In the absence of BA expression of *msr*1 is higher in *zea*3.1 than in the wild type, suggesting that the mutant may be more sensitive to endogenous hormones.

However, the cytokinin and auxin concentrations in 3-week-old seedlings remained unchanged in the mutant, suggesting that the *zea*3.1 mutation alters the response to cytokinins, not by modifying the endogenous levels of cytokinins or auxin but rather by affecting the sensitivity to these hormones. Together, these data suggest that ZEA3 normally represses the cytokinin response in wild-type *N. plumbaginifolia* seedlings and that repression is relieved in the mutant, thereby increasing its sensitivity and amplitude of response. Thus, the *zea*3.1 mutant, which was originally classified as cytokinin-resistant because it showed cotyledon expansion on high cytokinin concentrations (Faure et al., 1994), actually exhibits an increased sensitivity to cytokinin. This discrepancy is explained by the fact that *zea*3.1 displays not only a higher cytokinin sensitivity but also an amplified response. In particular, cotyledon expansion, which is a marginal cytokinin response in wild-type seedlings, is so enhanced in *zea*3.1 that it leads to an apparent growth at a high cytokinin concentration.

BA-induced expansion of cotyledons is a reliable cytokinin assay in plants such as radish, cucumber, and *N. plumbaginifolia* (Thomas et al., 1981; Nogué et al., 1995). Cotyledon growth has been shown to be primarily due to cell enlargement caused by wall loosening and water uptake (Thomas et al., 1981; Rayle et al., 1982). Hypertrophy of cotyledons in *zea*3.1 in response to cytokinins is mainly due to stimulation of cell division. This is consistent with the known role of cytokinins in promoting cell division in combination with auxin in cell culture (for review, see Jacqmard et al. [1994]). A restraint on cell division might be relieved in the mutant in response to cytokinins by a modification of the sensitivity to the normal auxin to cytokinin ratio leading to cell proliferation.

Unlike cotyledon expansion, the swelling of the hypocotyl induced by BA is associated only with cell enlargement. This is characteristic of an ethylene response. Similar results due to cytokinin stimulation of ethylene production were obtained in mung bean hypocotyls and Arabidopsis seedlings (Yip and Yang, 1986; Cary et al., 1995). In accordance with this hypothesis, we found that *zea*3.1 seedlings grown on 2(aminoethoxyvinyl)-glycine (AVG), an ethylene biosynthesis inhibitor, did not show cytokinin-induced hypocotyl enlargement but maintained cotyledon expansion. Cytokinin-dependent ethylene production might thus be considered another cytokinin response that is amplified in *zea*3.1. It may be that ZEA3 modulates other hormone-signaling/response pathways, such as those for auxin or ethylene, because some aspects of the mutant response are reminiscent of auxin (cell division) or ethylene (hypocotyl enlargement) effects. It would be of interest to analyze auxin regulation of *msr*1 expression in *zea*3.1 along with other auxin responses and to investigate whether auxin responses are also altered in the mutant.

ZEA3 could modulate cytokinin sensitivity in seedlings in interaction with other signal-transduction components such as protein kinases or phosphatases. Inhibitor studies have shown that protein phosphorylation plays some role in auxin and cytokinin regulation of *msr*1 expression. In *N. plumbaginifolia* cell cultures hormone-induced *msr*1 mRNA accumulation is prevented by staurosporin, a protein kinase inhibitor, and stimulated by okadaic acid, a phosphatase 1 and 2A inhibitor (Dominov et al., 1992). On the contrary, in wild-type seedlings staurosporin induces *msr*1 expression, whereas okadaic acid has no effect. In *zea*3.1 staurosporin increases *msr*1 expression and okadaic acid slightly decreases it. The additive effects of the *zea*3.1 mutation and staurosporin on *msr*1 induction implies that ZEA3 and the staurosporin-sensitive protein kinase corepress *msr*1 gene expression. Two models can be proposed, depending on the nature of *zea*3.1 mutation. If *zea*3.1 is a null mutation, ZEA3 and the staurosporin-sensitive kinases act independently on separate pathways to repress *msr*1 expression. But if *zea*3.1 is a leaky mutation, the

Table I. *Cytokinin and auxin levels in 3-week-old wild-type and zea3.1 seedlings*

Each value is given in pmol/g dry weight and is an average (SD) of at least three experiments.

Cytokins and Auxins	Wild Type	*zea*3.1
IAA	232 (5.4)	262 (134)
Isopentenyladenine	1.6 (0.8)	0.7 (0.5)
Isopentenyl adenosine	0.7 (0.2)	1.2 (0.4)
Zeatin	nd[a]	nd
Glucopyranosyl-zeatin	0.7 (0.1)	1.8 (1.3)
Zeatin riboside	0.7 (0.1)	0.7 (0.2)

[a] nd, Not detectable.

staurosporin-sensitive kinases might act in the same regulatory pathway as ZEA3.

The cytokinin responses in *N. plumbaginifolia* reported here differ from previous reports. The nature of cotyledon expansion or the regulation of *msr1* gene expression by protein phosphorylation are different in seedlings compared with cells in culture or detached organs (Thomas et al., 1981; Rayle et al., 1982; Dominov et al., 1992). These differences might be due to differences in experimental systems. In cell cultures or in detached organs, cells are either proliferating or senescing and may have a different hormonal status. The use of intact seedlings is a means of maintaining normal interactions between growth regulators and plant cells and should ensure the preservation of cytokinin metabolism and transport.

To explain cytokinin responses observed in *zea*3.1, we propose that ZEA3 could act as a modulator of cytokinin-transduction/response pathways repressing cytokinin responses. The involvement of the *zea*3 mutation in the control of primary metabolism and cotyledon development (Faure et al., 1994) suggests that *zea*3 encodes for a broader regulatory function integrating cytokinin response and maybe other hormones into seedling development.

ACKNOWLEDGMENTS

We gratefully acknowledge Steve H. Howell (Cornell University, Ithaca, NY) for helpful discussions and for providing the *msr1* probe and Magda Bonnet (University Paris VI) for great help with hormone measurements. We thank Michel Laloue (Institut National de la Recherche Agronomique, Versailles, France) for providing cytokinin analogs. We are grateful to Annette Ross and Michael Burnet for critical readings of the manuscript, and we wish to thank Jean-Marie Pollien and Krystyna Gofron for taking care of the plants.

Received January 17, 1997; accepted April 21, 1997.

LITERATURE CITED

Baskin TI, Cork A, Williamson RE, Gorst JR (1995) *STUNTED PLANT1*, a gene required for expansion in rapidly elongating but not dividing cells and mediating root growth responses to applied cytokinin. Plant Physiol **107:** 233–243

Binns AN (1994) Cytokinin accumulation and action: biochemical genetic and molecular approaches. Annu Rev Plant Physiol Plant Mol Biol **45:** 197–209

Bitoun R, Rousselin P, Caboche M (1990) A pleiotropic mutation results in cross-resistance to auxin, abscisic acid and paclobutrazol. Mol Gen Genet **220:** 234–239

Blonstein AD, Parry AD, Horgan R, King PJ (1991) A cytokinin resistant mutant of *Nicotiana plumbaginifolia* is wilty. Planta **183:** 244–250

Boutry M, Chua NH (1985) A nuclear gene encoding the beta subunit of the mitochondrial ATP synthase in *Nicotiana plumbaginifolia*. EMBO J **4:** 2159–2165

Caboche M (1987) Nitrogen, carbohydrate and zinc requirements for the efficient induction of shoot morphogenesis from protoplast derived colonies of *Nicotiana plumbaginifolia*. Plant Cell Tissue Org Cult **8:** 197–206

Cary AJ, Liu W, Howell SH (1995) Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. Plant Physiol **107:** 1075–1082

Chaudhury AM, Letham DS, Craig S, Dennis ES (1993) *amp1*—a mutant with high cytokinins and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J **4:** 907–916

Chory J, Aguilar N, Peto CA (1991) The phenotype *of Arabidopsis thaliana det1* mutants suggests a role for cytokinins in greening. Symp Soc Exp Biol **45:** 21–29

Chory J, Reinecke S, Sim S, Washburn T, Brenner M (1994) A role for cytokinins in de-etiolation in *Arabidopsis*. Plant Physiol **104:** 339–347

Davies PJ (1995) Hormones in tissue culture and propagation. *In* PJ Davies, ed, Plant Hormones. Kluwer Academic, Dordrecht, The Netherlands, pp 13–38

Deikman J, Ulrich M (1995) A novel cytokinin-resistant mutant of *Arabidopsis* with abbreviated shoot development. Planta **195:** 440–449

Dias M, Mornet R, Laloue M (1995) Synthesis, azido-tetrazole equilibrium studies and biological activity of 1-(2-azido-6-chlorpyrid-4-yl)-3-phenylurea, a photoaffinity labeling reagent for cytokinin-binding proteins. Bioorg Med Chem **3:** 361–366

Dominov JA, Stenzler L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* by feedback regulation. Plant Cell **4:** 451–461

Faure J-D, Jullien M, Caboche M (1994) *Zea3*: a pleiotropic mutation affecting cotyledon development, cytokinin resistance and carbon-nitrogen metabolism. Plant J **5:** 481–491

Haystead TAJ, Sim ATR, Carling D, Honnor RC, Tsukitani Y, Cohen P, Hardie DG (1989) Effects of the tumor promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature **337:** 78–81

Jacqmard A, Houssa C, Bernier G (1994) Regulation of the cell cycle by cytokinins. *In* DWS Mok, MC Mok, eds, Cytokinins: Chemistry, Activity and Function. CRC, Boca Raton, FL, pp 197–215

Julliard J, Sotta B, Pelletier G, Miginiac E (1992) Enhancement of naphthaleneacetic acid-induced rhizogenesis in Ti-DNA-transformed *Brassica napus* without significant modification of auxin levels and auxin sensitivity. Plant Physiol **100:** 1277–1282

Jullien M, Lesueur D, Laloue M, Caboche M (1992) Isolation and preliminary characterization of cytokinin-resistant mutants in *Nicotiana plumbaginifolia*. *In* M Kaminek, DWS Mok, E Zazimalova, eds, Physiology and Biochemistry of Cytokinins in Plants, Proceedings of the International Symposium on Physiology and Biochemestry of Cytokinins in Plants. SPB Academic, The Hague, pp 157–162

Kay R, Chan A, Daly M, McPherson J (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science **236:** 1299–1302

Kraepiel Y, Marrec K, Sotta B, Caboche M, Miginiac E (1995) *In vitro* morphogenic characteristics of phytochrome mutants in *Nicotiana plumbaginifolia* are modified and correlated to high indole-3-acetic acid levels. Planta **197:** 142–146

Krikorian AD (1995) Hormones in tissue culture and propagation. *In* PJ Davies, ed, Plant Hormones. Kluwer Academic, Dordrecht, The Netherlands, pp 774–796

Mok MC (1994) Cytokinin in plant development-an overview. *In* DWS Mok, MC Mok, eds, Cytokinins: Chemistry, Activity and Function. CRC, Boca Raton, FL, pp 155–166

Nogué F, Jullien M, Mornet R, Laloue M (1995) The response of a cytokinin resistant mutant is highly specific and permits a new cytokinin bioassay. Plant Growth Regul **17:** 87–94

Orbovic V, Kieu K (1996) A semi-automatic method for measurement of seedling length. Plant Growth Regul **20:** 303–305

Parry AD, Blonstein AD, Babiano MJ, King PJ, Horgan R (1991) Abscisic acid metabolism in a wilty mutant of *Nicotiana plumbaginifolia*. Planta **183:** 237–243

Rayle DL, Ross CL, Robinson CW (1982) Estimation of osmotic parameters accompanying zeatin-induced growth of detached cucumber cotyledon. Plant Physiol **70:** 1634–1636

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Santoni V, Bellini C, Caboche M (1994) Use of two-dimensional protein-pattern analysis for the characterization of *Arabidopsis thaliana* mutants. Planta **192:** 557–566

Su W, Howell SH (1992) A single genetic locus, *Ckr1*, defines *Arabidopsis* mutants in which root growth is resistant to low concentrations of cytokinin. Plant Physiol **99:** 1569–1574

Thomas J, Ross CW, Chastain CJ, Koomanoff N, Hendrix JE, van Volkenburgh E (1981) Cytokinin induced cell wall extensibility in excised cotyledons of radish and cucumber. Plant Physiol **68:** 107–110

Wang TL (1994) Cytokinin mutants. *In* DWS Mok, MC Mok, eds, Cytokinins: Chemistry, Activity and Function. CRC, Boca Raton, FL, pp 255–268

Yip W, Yang SF (1986) Effect of thidiazuron, a cytokinin-active urea derivative, in cytokinin-dependent ethylene production systems. Plant Physiol **80:** 515–519