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RESEARCH ARTICLE

Pea PR 10.1 is a ribonuclease and its transgenic expression elevates cytokinin levels

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Abstract The constitutive expression of a cDNA encoding a pea (*Pisum sativum* L.) PR 10 protein in *Brassica napus* leading to an enhancement of germination under saline conditions has been previously reported. In order to understand the biochemical function of this pea PR 10 protein, its cDNA has been expressed in *Escherichia coli* and the recombinant protein purified to homogeneity. Ribonuclease activity of the recombinant pea PR 10 protein has been demonstrated for the first time using an in-solution as well as an in-gel RNA degradation assay. Furthermore, in order to characterize the changes brought about as a result of the constitutive expression of the pea PR 10 cDNA in *B. napus*, we have measured the endogenous concentrations of several phytohormones. Increased cytokinin and, decreased abscisic acid (ABA) were observed in 7-day-old transgenic seedlings whereas no significant changes in the concentrations of gibberellin (GA) or indole-acetic acid (IAA) were observed at this stage of growth and development. The potential role(s) of PR 10 proteins with RNase activity and elevated cytokinins during plant stress responses as well as the possible relationship between PR 10 protein and changes in cytokinin concentrations are discussed.

Keywords *Brassica napus* · Cytokinin · Pathogenesis related (PR) 10 · Ribonuclease · tRNA

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Introduction

Plant pathogenesis-related (PR) proteins are, as the name suggests, expressed in response to pathogen challenge as well as during exposure of plants to abiotic stresses. They can be divided into at least 14 different families on the basis of their biological activity and are mostly secreted proteins (van Loon et al. 1994). A notable exception to this rule are the proteins belonging to the class 10 family of PR proteins, the PR 10 protein family (van Loon et al. 1994). PR 10 proteins are small (15–18 kDa), acidic, active in the cytosol and possess similar three-dimensional structures even though there may be significant differences at the level of their primary

structures (van Loon et al. 1994; Markovic-Housely et al. 2003). Even though PR 10 proteins have been detected in a number of plant species and are known to be induced by pathogens or abiotic stresses, their precise biological activities are currently unknown.

Based on similarities in primary structures between plant PR 10 proteins and a ribonuclease from ginseng, it has been suggested that these proteins may be RNases involved in plant defense (Moiseyev et al. 1994). This proposal has had some support due to the demonstrated RNase activity of some PR 10 proteins (Bantignies et al. 2000; Wu et al. 2003). However, the proposed function of PR 10 proteins as RNases involved in plant defense has been questioned due to the expression of PR 10 genes during normal growth and development of plants (Liu et al. 2003 and references therein). Moreover, researchers have also shown that PR 10 proteins bind many types of molecules including cytokinins, brassinosteroids, fatty acids and flavonoids (Fujimoto et al. 1998; Mogensen et al. 2002; Markovic-Housely et al. 2003) suggesting that all PR 10 proteins may not be RNases involved in plant defense. Thus, despite an increasing number of studies aimed at characterizing the role(s) of PR 10 proteins in plant processes during normal growth and development as well as during pathogen challenge, our understanding of their biological activities is far from being complete.

As mentioned earlier, in addition to pathogen-induced expression of PR 10 genes, abiotic stresses including salinity can also induce their expression suggesting a possible role in ameliorating the deleterious effects of stress (Ekramoddoullah et al. 1998; Hashimoto et al. 2004; Kav et al. 2004). Such a role is further supported by our observation that the constitutive expression of a pea PR 10 gene in *B. napus* enhances their germination and early seedling growth under saline conditions (Srivastava et al. 2004). However, the precise biochemical activities of pea PR 10 proteins as well as the mechanism(s) by which the pea PR 10 gene was able to enhance germination of *B. napus* are currently unknown. In this article, we describe the overexpression of pea PR 10.1 cDNA in *Escherichia coli*, the purification of the recombinant protein and demonstrate that this protein possesses RNase activity. In order to further characterize the differences between the transgenic and wild type *B. napus* seedlings, we have also determined the endogenous concentrations of several phytohormones in both seedlings. The potential role(s) of pea PR 10 proteins with RNase activity and the observed changes in phytohormone concentrations in mediating plant responses to abiotic stress are discussed.

Materials and methods

Overexpression and purification of pea PR 10. 1 in *E. coli*

PR 10.1 cDNA was amplified using two specific primers; 5′-GTG GTC GCA TAT G**GA AAA TTT GTA CTT TCA AGG T**AT GGG TGT TTT TAA TGT TGA AGA TGA AAT CAC TTC TG-3′ with a *Nde*I (underlined) and rTEV protease (bold) sites and 5′-TAT ATA GCT CGA GTT AGT TGT AAT CAG GAT GAG CCA AAC AGT AAC C-3′ with a *Xho*I site (underlined). Amplified products were digested with *Nde*I and *Xho*I (New England Biolabs, ON, Canada) and cloned into pET28a bacterial expression vector (Novagen, WI, USA) for the expression of an N-terminal poly-histidine-tagged fusion protein and used to transform *E. coli* Rosetta (DE3) expression cells (Novagen). PR 10.1 protein was induced as recommended by the manufacturer (Novagen) using 1 mM IPTG, at RT for 3 h. Bacterial cells were harvested by centrifugation at 8300 × *g* for 10 min at 4°C and the pellet resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, 1% Triton X100, complete EDTA-free protease inhibitor cocktail (Roche Inc., USA), pH 7.5].

Cells were lysed using a SONIC 300 Dismembrator (Artek Systems Corp., NY, USA) with 10 cycles of 30 s each at a setting of 0.8 relative output with 1 min cooling step (on ice) between each cycle. After centrifugation (13,800 × *g*, 10 min, 4°C), the supernatant was applied to a Ni-NTA agarose (Qiagen) column equilibrated with lysis buffer. The column was washed with 20 column volumes of wash buffer 1 (50 mM NaH_2 PO_4, 300 mM NaCl, 5 mM β-mercaptoethanol, 1% Triton X100, 10% glycerol, 15 mM imidazole, complete EDTA-free protease inhibitor cocktail, pH 7.5) and then with 15 column volumes of wash buffer 2 (same as buffer 1 but with 30 mM imidazole) to remove weakly bound bacterial proteins. The PR 10.1 protein was eluted from the column with wash buffer 3 (same as buffer 1 with

150 mM imidazole). The eluted, histidine-tagged protein was dialyzed overnight against rTEV protease buffer (50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT) at 4°C. The histidine tag was removed by incubation with 1.2 U/μg of the rTEV protease (Invitrogen, CA, USA) for 5 h at 30°C followed by an overnight incubation at 4°C and subsequent chromatography on Ni-NTA agarose column and collection of the flow through.

RNase activity measurements

In-solution RNase activity assays with the purified, recombinant PR 10.1 was performed as described by Bantignies et al. (2000) with modifications. Reaction mixtures containing 6 μg total RNA from either pea or canola tissue and 3, 6 or 9 μg uncleaved or cleaved proteins in rTEV buffer (see above) were incubated at RT for 3 h, extracted with equal volume of phenol–chloroform and the aqueous layer was analyzed on a 1.2% agarose gel. RNA was isolated from the roots of 7-day-old pea seedlings or from the shoots of 2-week-old canola plants as described earlier (Srivastava et al. 2004).

In-gel RNase activity assays with recombinant pea PR 10.1 as well as with crude, pea seedling root extracts were performed as described by Yen and Green (1991). The glass plates were treated with 0.1% DEPC overnight and autoclaved. The gel casting units were treated with RNase Zap (Ambion Inc., USA). The 15% acrylamide gel was cast with 2.4 mg/ml yeast tRNA and recombinant or crude protein extract was mixed with gel loading dye and loaded in the well without boiling. The protein separation was performed in a Mini PROTEAN 3 vertical slab system (Bio-Rad) at constant voltage (160 V) until the dye front reached the bottom of the gel. After electrophoresis the gels were washed twice with 25% isopropanol (v/v) in 0.01 M Tris–HCl for 10 min each at RT (20 ± 2°C) to remove SDS and renature the protein. The gels were subsequently washed twice with 0.01 M Tris–HCl for 10 min each at RT (20 ± 2°C) to remove the isopropanol. Following the washing steps, gels were incubated in 0.1 M Tris–HCl at 51°C for 50 min to allow the RNases to renature and digest the tRNA in the gel. Gels were stained with 0.2% (w/v) toluidine blue O (Aldrich Inc., USA) in 0.01 M Tris–HCl for 10 min at RT and destained by incubating twice with 0.01 M Tris–HCl for 10 min each after which the gels were rinsed and stored in 10% glycerol–0.01 M Tris–HCl.

Phytohormone analysis

Transformation of canola (*Brassica napus* cv. Westar) with the pea PR 10.1 cDNA and its constitutive expression in the transgenic line GN1-5#22 has been previously described (Wang et al. 1999; Srivastava et al. 2004). Westar and transgenic seeds (from T_4 plants) were germinated and grown for 7 days under aseptic conditions in sterile Petri dishes (Srivastava et al. 2004) and the entire seedlings were freeze-dried for phytohormone analysis. Fresh 7-day-old *B. napus* (transgenic and Westar) tissue was collected and immediately frozen in liquid N_2 and later freeze-dried. The freeze-dried tissue was ground in liquid nitrogen and extracted with 80% methanol. [$^2H_6$] ABA (250 ng), [$^{13}C_6$] IAA (200 ng) and 20–33 ng each of [$^2H_2$] GA_1 and [$^2H_2$] GA_{20} were added to the aqueous MeOH extracts as internal standards. The extract was then subjected to reversed phase C_{18} HPLC separation followed by identification and quantification analysis on gas chromatograph connected to a mass spectrometer (GC–MS; Agilent 6890) using the selected ion monitoring (SIM) mode, as described in Fellner et al. (2001). Quantification was accomplished by reference to the stable isotope-labeled internal standard using equations for isotope dilution analysis, adapted by DW Pearce (see Jacobsen et al. 2002) from Gaskin and MacMillan (1991). For CK analysis, 7-day-old *B. napus* seedlings were extracted, purified and quantified by LC-(+) ESI-MS/MS using the isotope dilution method as described in Ferguson et al. (2005). One hundred nanograms of the following were added as internal standards: [$^2H_6$]iP, [$^2H_6$][9R]iP, *trans*-[$^2H_5$]Z, [2H3]DZ, *trans*-[$^2H_5$][9R]Z, [$^2H_3$][9R]DHZ, [$^2H_6$][9RMP]iP, *trans*-[$^2H_5$][9RMP]Z and [$^2H_3$] [9RMP]DHZ (OlChemIm Ltd., Olomouc, Czech Republic).

Results and discussion

The recombinant protein was purified from the soluble fraction by chromatography on Ni-NTA column (Fig. 1). RNase activities of recombinant pea PR 10.1, with or without the histidine tag, were determined using an in-solution as well as an in-gel RNA degra-

dation assay (Fig. 2). It was evident that both forms of the protein possessed RNase activity capable of hydrolyzing pea and canola RNA (Fig. 2a, b). In order to confirm that the PR 10.1 protein is a RNase, we performed in-gel RNase assays as described by Yen and Green (1991). These in-gel assays produce gels with a dark background and proteins with RNase activities are detected on the basis of the clear region observed at molecular weights corresponding to the protein(s) of interest. Results shown in Fig. 2c clearly demonstrate that both forms of recombinant PR 10.1 protein (with or without the histidine tags) possess RNase activity. Also included in Fig. 2c is a crude pea seedling root protein extract where RNase activity is observed at the molecular weight (~16 kDa) corresponding to PR 10 proteins indicating that the native proteins also possess RNase activity.

As described earlier, PR 10 proteins are induced by both abiotic as well as biotic stresses. For example, infection with the pathogens *Fusarium solani* in pea (Fristensky et al. 1985), *Magnaporthe grisea* in Rice (McGee et al. 2001), *Rhynchosporium secalis* in barley (Steiner-Lange et al. 2003), *Cuscuta trifolii* in alfalfa (Borsics and Lados 2002) and *Cochliobolus heterostrophus* in sorghum (Lo et al. 1999) have been reported to induce PR 10 proteins. The ribonuclease activity of PR 10 proteins suggests a potential role in defense against pathogenic infections and it has been demonstrated that the constitutive expression of a pea PR 10 gene in potato conferred resistance to potato early dying disease (Chang et al. 1993). However, no resistance to the fungal pathogen *Leptosphaeria maculans* was observed in transgenic *B. napus* constitutively expressing the pea PR 10.1 gene (Wang et al. 1999). Recently, it was demonstrated that a capsicum PR 10 protein had RNase-mediated antiviral activity against Tobacco Mosaic Virus (TMV) which was enhanced by phosphorylation (Park et al. 2004). Although numerous articles report the elevation of PR 10 proteins by various abiotic stresses our previous finding that the constitutive expression of a pea PR 10 protein enhances germination under saline conditions remains the only report that PR 10 confers a positive effect on growth (Srivastava et al. 2004).

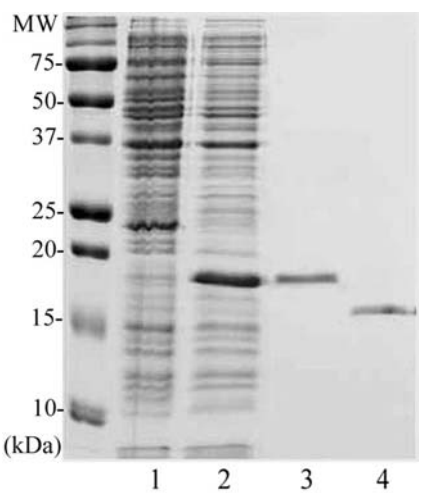

Fig. 1 Over expression and purification of pea (*P. sativum*) PR 10.1 in *E. coli*. Lanes 1, 2 are cell-free extracts from uninduced and induced *E. coli* cultures, respectively, lanes 3 and 4 are the purified, recombinant PR 10.1 proteins with or without the N-terminal poly-histidine tag, respectively

The constitutive expression of the pea PR 10.1 cDNA in *B. napus* minimizes the deleterious effects of salinity on root and shoot growth (Srivastava et al. 2004). In the absence of NaCl, there were no appreciable differences in the mean root lengths of the 7-day-old transgenic seedlings compared to wild type seedlings of a similar age whereas the mean shoot lengths were slightly higher (Srivastava et al. 2004). The appearance of the wild type and transgenic seedlings of different chronological ages is shown in Fig. 3. It is apparent that the radicles of the transgenic seedlings are better developed at 48 h when compared to the wild type. This may have resulted from the earlier (~12 h) germination of the transgenic seeds; although, both transgenic and wild type seeds had completed germination within the first 24 h (Fig. 3a). In the case of 7-day-old transgenic and wild type seedlings (6 days after germination) grown either in Petri dishes or soil, the observed differences were negligible which supports our previous observations that phenotypic differences of 7-day-old seedlings are minimal (Fig. 3b, c).

In order to further characterize the differences between the transgenic and wild type *B. napus* seedlings that may help in understanding the basis for the observed tolerance to salinity during early seedling growth (Srivastava et al. 2004), we investigated whether the changes were hormonally mediated. We determined the endogenous concentrations of four phytohormones groups in 7-day-old seedlings (Table 1). Seven-day-old seedlings were used for this investigation in order to obtain sufficient tissue to perform all the

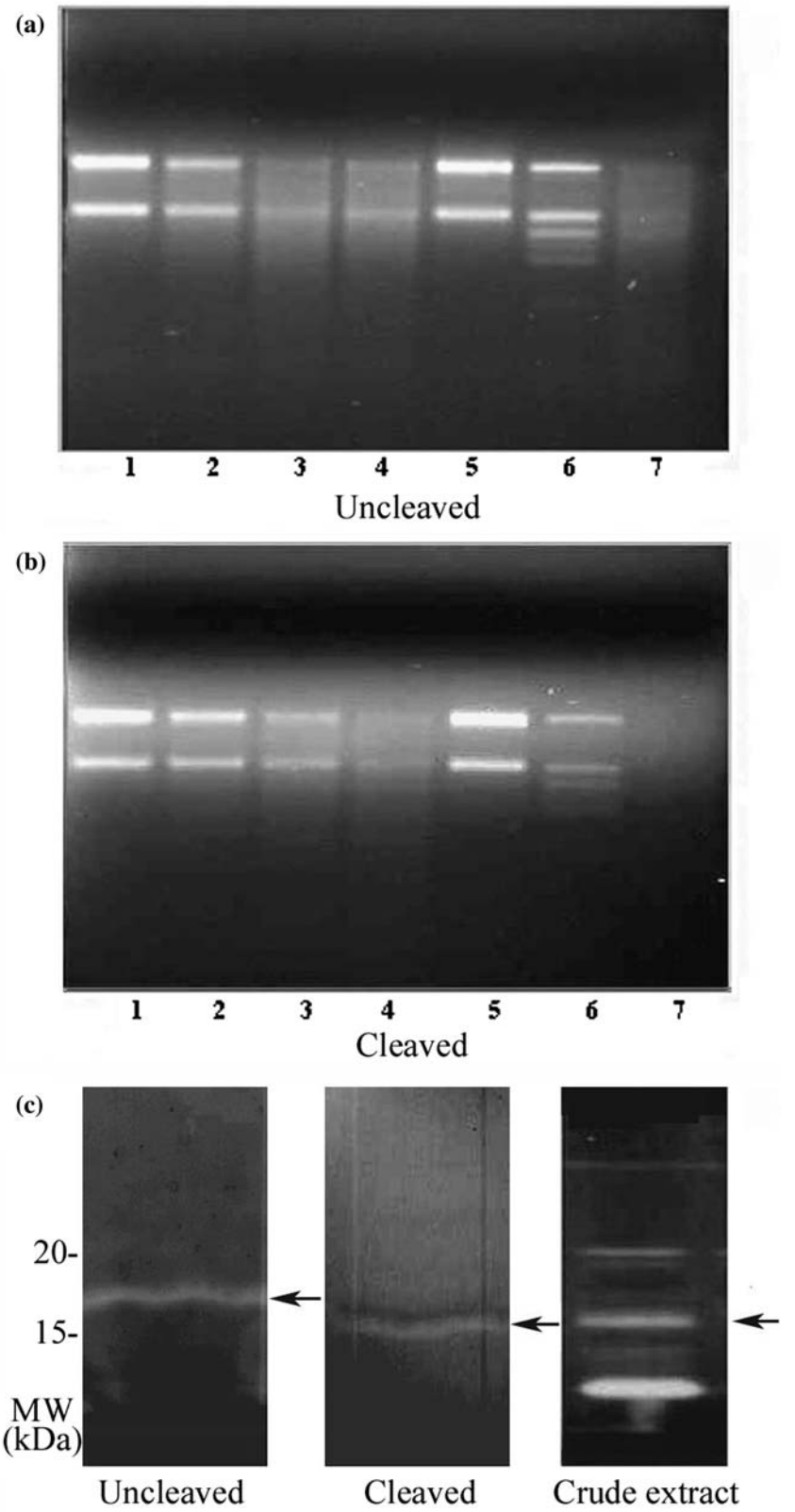

Fig. 2 Ribonuclease activity of pea (*P. sativum*) PR 10 protein. In-solution RNA degradation assay with histidine-tagged recombinant PR 10.1 (**a**) or recombinant PR 10.1 without the histidine tag (**b**). In both panels A and B, lanes 1–4 are reactions with total RNA from pea and 0, 3, 6 or 9 μg recombinant PR 10.1 protein; lanes 6 and 7 with total RNA from canola and 0 or 9 μg recombinant PR 10.1 protein. Lane 5 is total RNA from pea incubated with recombinant PR 10.1 protein (9 μg) which had been boiled in a boiling water bath for 15 min. In-gel RNA degradation assay (**c**); from left to right are purified, recombinant histidine-tagged protein, cleaved recombinant protein and crude pea seedling root extract. SDS-PAGE gels containing yeast tRNA are used for electrophoresis and protein bands with RNase activity are visualized by staining with toluidine blue O (Yen and Green 1991). Arrows point to the clear bands which are indicative of RNase activity

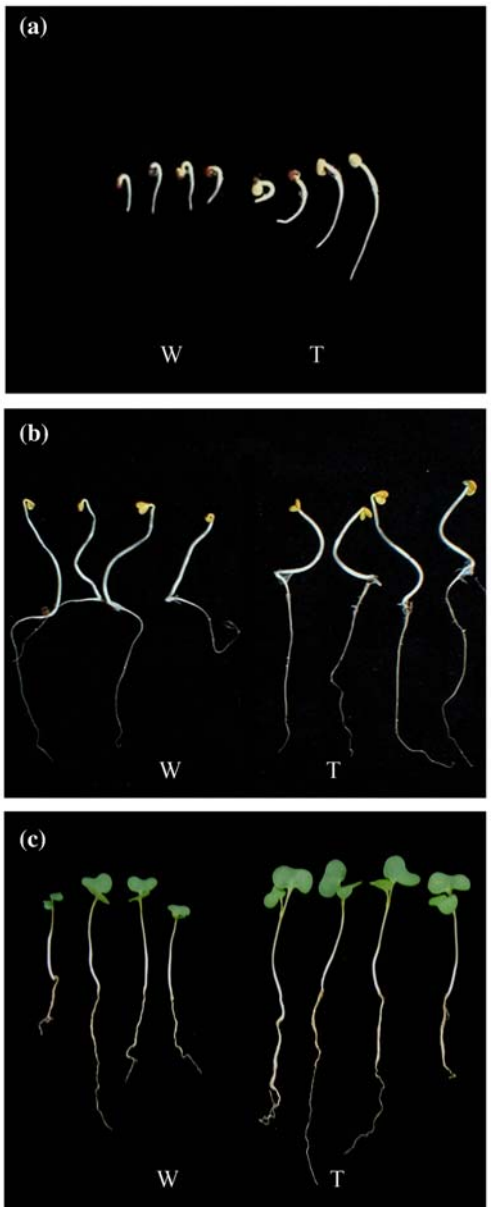

Fig. 3 Comparison of germination and early seedling growth of 7-day-old wild type and transgenic (pea PR 10.1) *B. napus* seedlings. Appearance of wild type (W) and PR 10.1 transgenic (T) seedlings after 2 days (**a**) and 7 days (**b**) after germination and growth in Petri dishes. (**c**) Appearance of 7-day-old wild type (W) and transgenic (T) seedlings in soil

phytohormone analyses as well as to ensure that there would be minimal developmental differences. No significant differences in IAA, GA_1 and GA_4 concentrations were observed whereas GA_9 could not be detected. The endogenous concentration of GA_{20} was higher in PR 10.1 seedlings (Table 1); however, since GA_{20} is not considered to be biologically active (Santes and Garcia-Martinez 1995), it is unlikely that this difference would have contributed to the increased salinity tolerance of the transgenic seedlings. However, the endogenous concentration of ABA showed almost a 2-fold decrease and cytokinin concentrations were higher in the transgenic seedlings (Table 1).

Abscisic acid (ABA) regulates a number of physiological processes in plants including the onset and maintenance of dormancy (Finkelstein et al. 2002; Gubler et al. 2005). The relationship between ABA and GA during germination is well known. For example, ABA delays or prevents seed germination and determines dormancy levels whereas GA breaks dormancy and promotes germination (Groot and Karssen 1992; Koornneef et al. 2002). Relationship between CK and ABA has also been reported in the literature including that by Chang et al. (2003) where transgenic petunia flowers with elevated cytokinin

Table 1 Endogenous concentrations of various hormones in 7-day-old wild type and transgenic (pea PR 10.1) *B. napus* seedlings

Hormone	Quantity (ng/g DW^{-1}) ($n=2$)	
	Wild type (Mean ± SE)	Transgenic (Mean ± SE)
GA1	0.57 ± < 0.01	0.48 ± 0
GA4	5.5 ± 0.9	5 ± 0.3
GA20	10.2 ± 1.1	19.2 ± 0.7
IAA	130 ± 7	119 ± 8
ABA	217 ± 4	106 ± 19
Cytokinins	pmol g DW^{-1} (n=2)	
Trans-Z	118 ± 0.2	273 ± 67
Cis-Z	Not detected	Not detected
iP	2 ± 0.8	0.9 ± < 0.1
Trans-[9R]Z	52 ± 18.4	4865 ± 2263
Cis-[9R]Z	32 ± 28	15 ± 5
[9R]DHZ	2.5 ± 0.9	58 ± 28
[9R]iP	2.7 ± 2.4	3.6 ± 0.7
Trans-[9RMP]Z	33 ± 5	1614 ± 701
Cis-[9RMP]Z	101 ± 19	101 ± 12
[9RMP]DHZ	10.3 ± 2.8	13.7 ± 4.9
[9R]iP	6.3 ± 0.3	6.3 ± 0.5
Cytokinin free bases (FB)	210 ± 7	5215 ± 2353
Cytokinin nucleotides (NT)	150 ± 26	1734 ± 718
Total cytokinins	360 ± 18	6949 ± 3071

concentrations, as a result of isopentenyl transferase (IPT) overexpression, significantly reduced the concentration of ABA. The observations of Chang et al. (2003) are consistent with our findings that the transgenic seedlings constitutively expressing the pea PR 10.1 cDNA have lower concentration of ABA compared to the wild type seedlings (Table 1). This reduction of ABA combined with the observed increases in cytokinin (CK) may contribute to the enhanced growth of the transgenic seedlings (see below).

A massive increase in the total cytokinin was observed in 7-day-old transgenic seedlings (Table 1). Changes in CK were comprised of the free base and ribosides (which are presumed to be active) as well as the nucleotide forms. More specifically, the biggest difference observed was a dramatic increase in the concentration of *trans*-zeatin riboside (*trans*-[9R]Z) and its nucleotide (*trans*-[9RMP]Z) in the transgenic seedlings. Much smaller increases were observed in the concentration of *trans*-zeatin and *trans*-[9RMP]DHZ in the transgenic seedlings (Table 1). No differences in the concentration of [9R]iP, iP or *cis*-[9R]Z were observed between the two types of seedlings whereas we were unable to detect *cis*-Z in either species (Table 1). Due to the large number (>1000) of seedlings required to perform the endogenous phytohormone analyses, the numbers in Table 1 reflect the average values from two independent extractions and analyses from seedlings that were pooled from several biological replicates.

Cytokinins are plant hormones that have been isolated from a number of species and, in germinating seedlings they are known to be synthesized by the embryonic axes (Villalobos and Martin 1992). Cytokinins may also be involved in post-germination processes such as root and hypocotyl growth (Singh and Sawhney 1992). Two de novo pathways for cytokinin biosynthesis have been demonstrated in plants (Kakimoto 2003) and are thought to be the major sources for endogenous, free cytokinins. The significance of such a large increase in CK is that they are thought to play an important role in mediating plant responses to stresses (Harding and Smigocki 1994; Gan and Amasino 1995). For example, it has been demonstrated that: CKs may function as antioxidants in germinating soybean seedlings (Gidrol et al. 1994), synthetic cytokinin analogues possess antioxidant activities (Brathe et al. 2002), and CKs can induce a metallothionein gene during copper stress (Thomas et al. 2005). The fact that elevated concentrations of CK may be contributing to the enhanced growth of transgenic seedlings under saline conditions is further supported by the fact that the greatest increases in the PR 10.1 transgenic seedlings were in *trans*-[9R]Z, one of the active CK in many known bioassays, and another potentially active form, *trans*-[9RMP]Z (Emery and Atkins 2005).

Combined with the stress-induced expression of plant PR 10 proteins, a possible implication is that PR 10 proteins mediate their functions through altering endogenous cytokinin concentrations. In fact, it is possible that the observed RNase activity of the PR 10 proteins may be responsible for the increase in cytokinin concentrations as, in addition to de novo biosynthetic pathways, cytokinins may also be derived from modified bases that occur in plant tRNAs (Haberer and Kieber 2002; Skoog and Armstrong 1970). Furthermore, tRNA has been demonstrated to be a source of cytokinin and it is possible that their turnover contributes to the pool of free, active cytokinins contributing as much as 40–50% of cytokinin pools (Barnes et al. 1980; Letham and Palni 1983; Taller et al. 1987; Prinsen et al. 1997). However, the isomer profile of CK detected in this study are not consistent with this hypothesis. Since the major CK moiety present in plant tRNAs is *cis*-Z (Taller 1994) and the degradation of tRNA by PR 10.1 would be expected to enhance the concentration of *cis*-Z, *cis*-[9R]Z and *cis*-[9RMP]Z. Our results show (Table 1), that there were no differences in the concentration of *cis*-[9RMP]Z, a reduction in *cis*-[9R]Z and *cis*-Z could not be detected. The possibility remains that that both *cis*-[9R]Z as well as *cis*-Z were converted to their respective *trans* forms by a *cis–trans* isomerase, like that which has been isolated from *Phaseolus vulgaris* endosperms (Bassil et al. 1993; Mok and Mok 2001). Moreover, *trans*-Z has been detected in the tRNAs of hop plants (*Humulus lupulus* L.) where it constituted 25% of total tRNA zeatin (Wang 1994). However, neither *P. vulgaris* nor hop plants belong to the same family as *B. napus* and the presence of an isomerase and/or *trans*-Z (or its nucleotide forms) in *B. napus* tRNA must be investigated. Another issue that warrants discussion at this stage is the fact that the concentration of the active *trans*-Z in the transgenic seedlings were only ~2-fold higher whereas the

concentrations of both *trans*-[9R]Z and *trans*-[9RMP]Z were at least 30-fold higher (Table 1). This may be due to the fact that *trans*-[9R]Z can be converted to either [9R]-DHZ or to *trans*-[9RMP]Z (Takei et al. 2004). It should be stressed that the endogenous CK concentrations were determined at a specific developmental stage (6 days after germination) which will, undoubtedly, have a significant effect on the proportion of individual species that are present.

In conclusion, although it is tempting to speculate that the RNase activity of pea PR 10 protein may lead to the enhanced endogenous cytokinin pool in the transgenic *B. napus*, it is equally possible that the PR 10 protein mediates the increase in cytokinins by some other mechanism including increased de novo biosynthesis or reduced degradation/oxidation. Alternatively, the differences in endogenous CK concentrations may be caused by subtle differences in seedling development although both wild type and transgenic seedlings used for phytohormone analyses were at 6 days after germination. To rule out this possibility, such hormone analyses must be performed encompassing a window of several days during germination and early seedling growth. To unequivocally test the role of RNase in elevating cytokinins in transgenic seedlings we have initiated these as well as additional experiments that include the feeding of radiolabelled tRNA (to determine the incorporation of the radiolabel in individual species of cytokinins) and the detection of any active *cis–trans* isomerase in *B. napus*. We are also in the process of expressing other pea PR 10 homologues in *Arabidopsis thaliana* in order to investigate the effects of PR 10 on cytokinin biosynthesis, degradation, conjugation, and signal transduction in a system with more comprehensive genomic resources.

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