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ORIGINAL RESEARCH

Expression Patterns of PR proteins with Different Extract Methods during Germination of Rape Seed (*Brassica napus* L.)

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Abstract This study was conducted to investigate the expression patterns of pathogenesis-related proteins (chitinase, β-1,3-glucanase and peroxidase) using activity staining of native-polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE during germination of rape seed (*Brassica napus* L. cv. Saturnin). The crude enzymes were extracted by distilled water (DW, pH 6.0) and 100 mM K-PO_4 buffer (pH 7.0). The expression patterns of chitinase isozymes changed clearly on 10% native-PAGE gel with DW and K-PO_4 buffer extract and on 12% SDS-PAGE gel with K-PO_4 buffer extract, except for 12% SDS-PAGE conducted using DW during germination. The active bands of the chitinase isozymes were observed as four major bands (ch1, ch2, 86, and 78 kDa) and three minor bands (71, 60, and 54 kDa) on 10% native-PAGE gel conducted using DW and K-PO_4 buffer extract. The two active bands on the 12% (w/v) SDS-PAGE gel presented as 34 and 29 kDa with DW extract, whereas one active band of 34 kDa was observed when the K-PO_4 buffer extract was used. Active bands of β-1,3-glucanase isozymes changed slightly on 10% native-PAGE gel with DW and K-PO_4 buffer extract during germination. The active band of β-1,3-glucanase isozymes were shown to have a high molecular weight (G1 and G2) on native-PAGE gel with DW extract at 0, 1, 2, and 3 days after germination, but not at 4 and 5 days. One active band of β-1,3-glucanase presented as G1 in the K-PO_4 buffer extract. Active staining of peroxidase was stronger earlier in the DW extract than K-PO_4 buffer extract at 2 days. The active bands showed as P1 and P2 in both DW and K-PO_4 buffer extract at 5 days after germination.

Keywords Chitinase · β-1,3-glucanase · Peroxidase · Germination · *Brassica napus* L.

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Introduction

Seeds belong to the dormancy condition in plant life, and embryos in seeds play an important role in the plant's entire growth stages. Respiration increases in response to various enzymes in seeds and storage nutrients become easily available for plant metabolism during germination. Many studies have been conducted to evaluate protein expression patterns during seed germination, and high molecular proteins are known to be degraded to low molecular proteins while the overall amount of specific proteins increases during germination.

Rape seeds are composed of oils (35–47%), proteins (15–32%), carbohydrates, fiber, ash, and vitamins. Mobilization of the major storage reserves in seeds is associated with the initial growth of the seedling. Bewley (1997) demonstrated that all of the cellular and metabolic events that are known to occur before the completion of germination of nondormant seeds also occur in imbibed dormant seeds.

Expression patterns of pathogenesis-related (PR) proteins are induced by hypersensitive reactions to pathogens (Punja and Zhang 1993), hardening and dehardening in winter wheat (Gaudet et al. 2000), and ethylene supplementation in bean leaves (Boller et al. 1983). Conversely, PR proteins are inhibited by accumulation of auxin and

cytokinin (Shinshi et al. 1987). PR proteins were first identified in tobacco leaves subjected to TMV treatments in the 1970's (Gianinazzi et al. 1970). Following the discovery of four PR proteins (PR-1a, 1b, 1c, and 2a) in tobacco leaves, different PR proteins have been identified in various plants (van Loon and van Kammen 1970). The activation of PR proteins was observed upon infection with pathogens such as virus, bacteria, and fungi under biotic stress conditions (van Loon 1985). In addition, the expression of PR proteins was found to increase under abiotic stress conditions such as osmotic stress and salt stress (Asselin et al. 1985; Dumas et al. 1987; King et al. 1986).

Generally, PR proteins are stable, soluble, and have resistance to proteolytic enzymes at low (around 3.0) pH levels (Stintzi et al. 1993). In addition, PR proteins exist as monomers of low molecular proteins in vocuole, cell membrane, and apoplasts in plants. At present, 14 PR families are officially recognized (van Loon and van Strien 1999). The PR proteins are primarily classified as chitinase, β-1,3-glucanase, and peroxidase. Many PR proteins studies have been conducted to evaluate the utilization and function of chitinase in plant growth (Quecine et al. 2008; Antimo et al. 2010). For example, chitinase was purified as a monomer of 25–35 kDa from the bean plant (Boller 1988). In addition, chitinases exist as endochitinase and produce chiti-oligosaccharide from chitin polymer. Activation of lysozyme hydrolyzes β-1,4-linkage of peptidoglycan composed of *N*-acetylmuramic acid and *N*-acetylglucosamine (Roberts and Selitrennikoff 1988).

Chitinase plays an important role in embryogenesis and during the initial formation of the cell membrane, and also acts as an antifreezing protein at low temperatures. In addition, hydrolytic enzymes such as chitinase and β-1,3-glucanase inhibit fungal growth in pea tissue (Mauch et al. 1988) and tobacco (Sela-Buurlage et al. 1993). β-1,3-Glucanase exists as a monomer of 25–35 kDa in plants, and this enzyme mainly produces glucose oligomer as endo-glucanase using substrates such as laminarin, unbranched β-1,3-glucan, and β-1,3-glucan (Meins et al. 1992). Specific substrates of β-1,3-glucanase have been observed in soybean and tobacco plants. In the case of soybean, the activity of β-1,3-glucanase was high for laminarin. In the case of tobacco, the activity of the enzyme was not different significantly when various substrates were used (Ham et al. 1991).

Many studies have shown that the expression patterns of chitinase and peroxidase are increased during germination and plant growth (Park et al. 1986; Bewley 1997). In addition, peroxidase activity has been shown to increase as an early response during infection of *Medicago truncatula* by *Rhizobium melitoti* (Cook et al. 1995) and infection of the tropical forage legume *Stylosanthes humilis* by *Colleto-trichum gloeosporioides* (Harrison et al. 1995). These proteins have been classified as PR9 with a type member of tobacco known as lignin-forming peroxidase and as ypr9 with gene symbols (Lagrimini et al. 1987). Welinder (1992) reported that peroxidases are involved in several plant defense responses including cell wall metabolism, wound healing, and auxin catabolism. Plant peroxidase produces antimicrobial phenolic compounds in the chemical defense systems against plant pathogens (Kobayashi et al. 1994).

The objective of this study was to investigate the expression patterns of PR proteins during the early germination stage of rape (*Brassica napus* L. cv. Saturnin) seeds with two different extract buffers.

Materials and methods

Preparation of rape seeds

Rape (*B. napus* L.) seeds were sterilized in 70% ethanol for 3 min, after which they were thoroughly washed with sterile distilled water. Crude enzymes of rape seeds (cultivar of Capitol, Pollen, and Saturnin) were extracted with 100 mM potassium phosphate (KH_2PO_4/K_2HPO_4) buffer. The three cultivars were used in an initial test to identify the pattern of chitinase activity (Fig. 1). In this study, the Saturnin cultivar was selected for further evaluation because of the diversity of chitinase active bands. Thirteen seeds of rape (*B. napus* L. cv. Saturnin) were then sown on filter paper on petri plates and allowed to germinate at 26°C in the dark. During germination, the seeds were sprayed with 5 ml distilled water once a day in the morning. Five seeds were collected at 0, 1, 2, 3, 4, and 5 days after seeding and then kept at −80°C for further assay.

Measurement of enzyme activity

Rape seeds were collected and then washed under running tap water. The seeds were then dried gently, after which they were ground with a mortar and pestle under liquid nitrogen. The ground samples were then homogenized in 100 mM potassium phosphate (KH_2PO_4/K_2HPO_4) buffer (pH 7.0) containing 2 mM ethylenediamine tetraaceticacid, 1% polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride, and distilled water (DW, pH 6.0). Next, the samples were centrifuged at 10,000×*g* and 4°C for 10 min, after which the supernatant was collected and stored at −20°C until use. The protein concentration was determined using the method described by Bradford (1976).

Chitinase activity was assayed by measuring the amount of the reducing end group, GlcNAc (*N*-acetyl-β-D-glucosamine), produced by swollen chitin (Lingappa and Lockwood 1962). The assay mixture consisted of 20 μl of enzyme extract, 500 μl of 0.5% swollen chitin

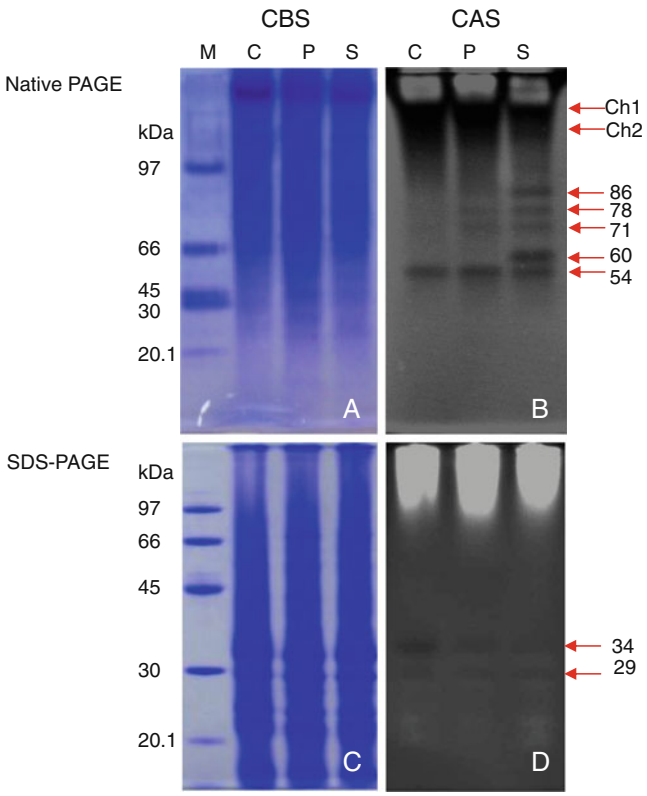

Fig. 1 Chitinase activity staining of crude enzymes extracted from rape seeds [Capitol (*C*), Pollen (*P*), and Saturnin (*S*)]. Native-PAGE 10% (w/v) gels (**a** and **c**), SDS-PAGE 12% (w/v) gels (**b** and **d**) stained with CBS (Coomassie brilliant blue R-250) and CAS (chitinase activity staining by a glycol chitin used as substrate and calcofluor white M2R used for resolution of dark bands). Protein marks (*M*), Capitol (*C*), Pollen (*P*), and Saturnin (*S*)

(Monreal and Reese 1969), and 480 μl of 50 mM sodium acetate buffer (pH 5.0). After incubation at 37°C for 1 h, 200 μl of 1 N NaOH was added. Next, the sample was briefly centrifuged (10,000×*g*, 5 min), after which 500 μl of supernatant was mixed with 1 ml of Schales' reagent (0.5 M sodium carbonate +1.5 M potassium ferricynide) and then heated in boiling water for 15 min. The absorbance was then immediately measured at 420 nm using a spectrophotometer (uQant Bio-Tek, USA). Finally, the activity was calculated based on comparison with a standard curve generated from known concentrations of GlcNAc. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour.

β-1,3-Glucanase activity was assayed by measuring the amount of the reducing end group, glucose, produced from laminarin (Yedidia et al. 2000). The assay mixture consisted of 20 μl of crude enzyme, 25 μl of 1% laminarin, and 455 μl of 50 mM sodium citrate buffer (pH 5.0). After incubation at 37°C for 1 h, 1.5 ml of 3-amino-5-nitrosalicylic acid (DNS) was added, and the sample was then heated in boiling water for 5 min. The absorbance was then immediately measured at 550 nm using a spectrophotometer. Next, the amount of reducing sugar was calculated using a standard curve generated from known concentrations of glucose. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per hour.

Peroxidase (POD) activity was assayed by measuring the amount of the reducing end group produced using the method described by Chance and Maehly (1955). The reaction mixture consisted 50 μl of 200 mM guaiacol, 2.8 ml of 10 mM phosphate buffer (pH 7.0), and 100 μl of enzyme extract. The reaction was started with 20 μl of 40 mM H_2O_2. The level of POD, which represented the oxidation of guaiacol, was determined based on the increase in absorbance at 470 nm over 1 min using a spectrophotometer (Mecasys, Optizen 3220UV, Korea).

Active staining by electrophoresis

After electrophoresis, 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel was stained with 0.12% Coomassie brilliant blue R-250. To evaluate the active staining of chitinase, 12% SDS-PAGE (containing 0.01% glycol chitin) was conducted according to the method described by Trudel and Asselin (1989) and native-PAGE 10% (w/v) was conducted according to the method described by Ornstein (1964). Briefly, the gel was incubated in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 and 1% skim milk at 37°C for 2 h with reciprocal shaking. A subsequent incubation was then conducted overnight under the same conditions, but without skim milk in buffer solution. The gel was then immersed in 500 mM Tris–HCl (pH 8.9) solution containing 0.01% calcofluor white M2R (Sigma F3397). The lysed zones were visualized and photographed using a UV transilluminator (Daihan Sci. Co., WGD-30, Korea).

For active staining of β-1,3-glucanase on 12% SDS-PAGE and 10% native PAGE gel containing 0.2% laminarin, the gel was immersed in 20 mM 2-(*N*-morpholino) ethansulfonic acid (MES) buffer (pH 6.0) containing 10 mM $CaCl_2$ for 15 min with the renewal of buffer every 5 min. The gel was then dipped in a 0.15% triphenyltetrazolium chloride solution containing 1 N NaOH, after which it was heated in a microwave for 2 min until red bands appeared. The gel was then treated with fixing solution (3% glycerol, 40% methanol, and 10% acetic acid) for detection of active bands (Pan et al. 1989).

For active staining of Guaiacol-peroxidase (GPOD) after 10% (w/v) native PAGE, the gels were soaked in 50 mM Tris buffer (pH 6.8) for 10 min, after which they were incubated with 0.46% (v/v) guaiacol and 13 mM H_2O_2 in the same buffer until red bands appeared. The gels were then fixed in water/methanol/acetic acid (6.5:2.5:1, v/v) (Caruso et al. 1999).

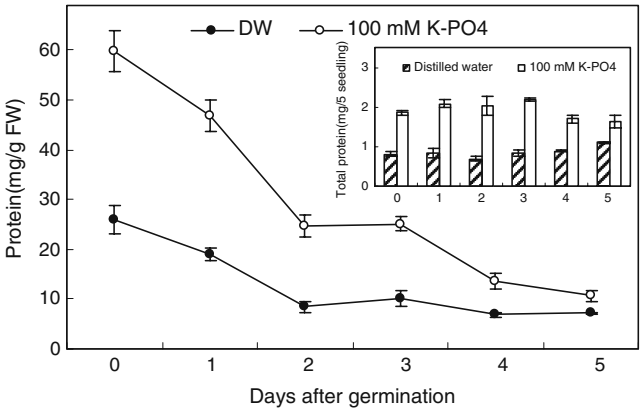

Fig. 2 Protein content of distilled water (*DW*) and 100 mM K-PO_4 extracts of rape (*B. napus* L. cv. Saturnin) seeds at 0, 1, 2, 3, 4, and 5 days after germination

Results

Chitinase active staining

Three types of oil rape (cv. Capitol, Pollen, and Saturnin) seeds were extracted with K-PO_4 buffer (pH 7.0) and the enzymes from the extract (protein 100 μg) were then loaded on 10% native-PAGE gel and 12% SDS-PAGE gel (Fig. 1). Chitinase isozymers in a Saturnin cultivar seed were shown as ch1, ch2, 86, 78, 71, 60, and 54 kDa on 10% native-PAGE gel and 34 and 29 kDa on 12% SDS-PAGE. The protein content of the rape (*B. napus* L. cv. Saturnin) seeds was assayed for different extraction with DW (pH 6.0) and 100 mM K-PO_4 buffer (pH 7.0) at 0, 1, 2, 3, 4, and 5 days after seed germination (Fig. 2). The protein content of the Saturnin seeds was 25.8 and 59.8 mg g^{-1} FW in the DW and K-PO_4 buffer extract, respectively, at day 0. The protein content of K-PO_4 buffer extract decreased rapidly by 58.8% at 2 days after germination. At 5 days after germination, the protein contents were decreased by similar levels of 7.2 and 10.6 mg/g FW in DW and K-PO_4 buffer extract. The total protein content did not differ significantly with time in DW or K-PO_4 buffer extract (Fig. 2, inner).

PR proteins activity

To evaluate the variety and evolution of the PR-protein activities, crude enzymes of rape (cv. Saturnin) seeds were assayed following extraction with DW (pH 6.0) and 100 mM K-PO_4 buffer (pH 7.0) at 0, 1, 2, 3, 4, and 5 days after seed germination (Fig. 3). The chitinase activity of Saturnin seeds was 0.92 and 0.54 unit/mg protein in DW and K-PO_4 buffer extract, respectively, at day 0 (Fig. 3a). The chitinase activity was decreased in K-PO_4 buffer extract during germination, while the chitinase activity was upregulated in DW extract at day 0, after which it rapidly decreased until day 5. The β-1,3-glucanase activity of Saturnin seeds was 4.5 and 1.9 unit/mg protein in DW and K-PO_4 buffer extract, respectively, at day 0 (Fig. 3b). The β-1,3-glucanase activity was increased in DW extract until day 3, after which it rapidly decreased until day 5, while that of K-PO_4 buffer increased slightly during germination. The peroxidase activity of Saturnin increased in both DW and K-PO_4 buffer extract during germination (Fig. 3c). Specifically, the peroxidase activity was 0.47 and

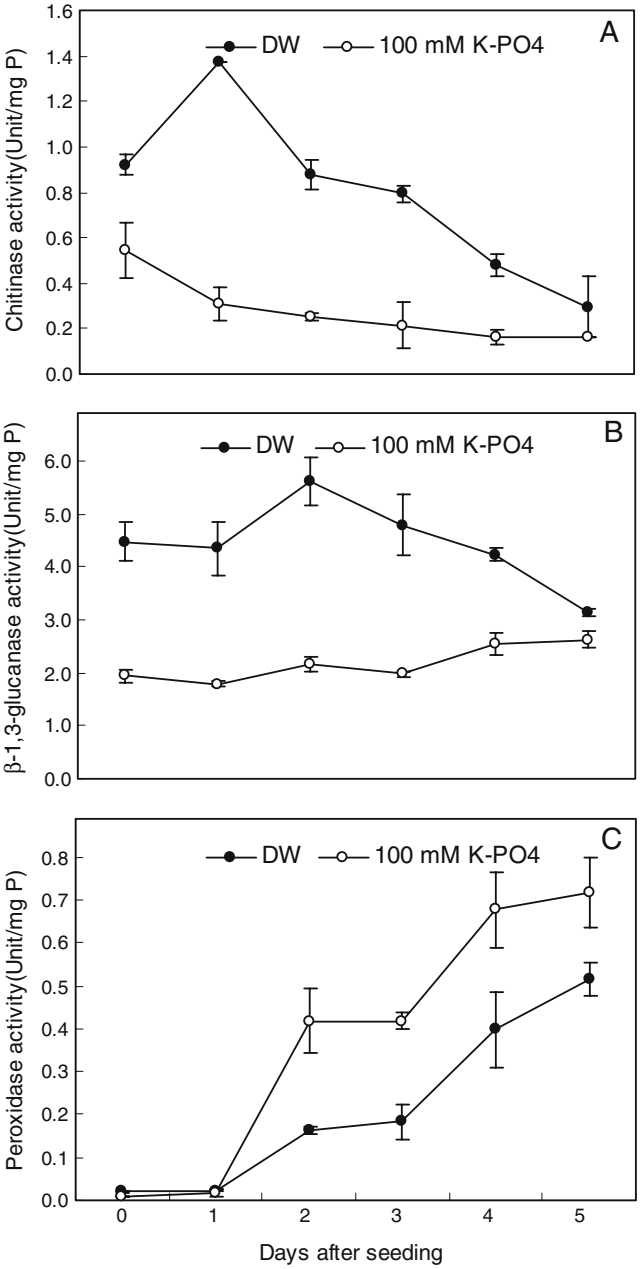

Fig. 3 PR protein (**a** chitinase, **b** β-1,3-glucanase, **c** peroxidase) activities following extraction of rape seeds (*B. napus* L. cv. Saturnin) with distilled water (*DW*) and 100 mM K-PO_4 at 0, 1, 2, 3, 4, and 5 days after germination

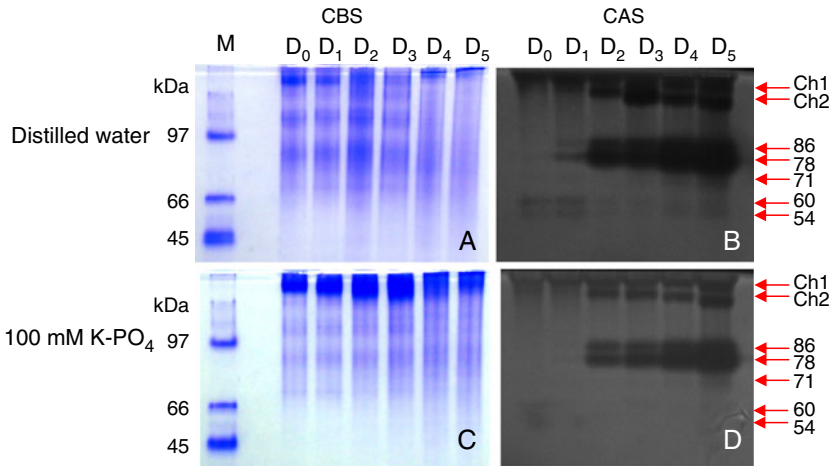

Fig. 4 Chitinase activity staining of rape (*B. napus* L. cv. Saturnin) crude proteins extracted with distilled water (**a** and **b**) and with 100 mM K-PO_4 buffer (**c** and **d**) were stained on native-PAGE 10% (w/v) gels with CBS (Coomassie brilliant blue R-250) and CAS (chitinase activity staining by a glycol chitin used as a substrate and calcofluor white M2R used for resolution of dark bands). Protein marks (*M*) and days (D_0, D_1, D_2, D_3, D_4, and D_5) after germination

0.65 units/mg protein in DW and K-PO_4 buffer extract, respectively, at 5 days after germination.

Gel electrophoresis

After native-PAGE, activity staining of chitinase was conducted to obtain profiles of these isozymes in Saturnin seeds (Fig. 4). The expression patterns of chitinase in rape (cv. Saturnin) seeds were observed on native-PAGE gel following extraction with DW (pH 6.0) and 100 mM K-PO_4 buffer (pH 7.0) at 0, 1, 2, 3, 4, and 5 days after seed germination. The chitinase active staining revealed seven isozyme bands (ch1, ch2, 86, 78, 71, 60, and 54 kDa) on native-PAGE gel (Fig. 4b). Chitin isozymes of ch1, ch2, 86, and 78 kDa were expressed strongly with time in both DW and K-PO_4 buffer extract, while that of 60 and 54 kDa was expressed at lower levels (Fig. 4b and d).

The expression patterns of chitinase in rape (cv. Saturnin) seeds were observed on SDS-PAGE gel following extraction with DW and 100 mM K-PO_4 buffer at 0, 1, 2, 3, 4, and 5 days after seed germination (Fig. 5). The active staining of chitinase in DW extract showed stronger 34 and 29 kDa bands than that of the K-PO_4 buffer extract (Fig. 5b and d).

The expression patterns of β-1,3-glucanase in rape (cv. Saturnin) seeds were observed on native-PAGE and SDS-PAGE gels following extraction with DW and 100 mM K-PO_4 buffer at 0, 1, 2, 3, 4, and 5 days after seed germination (Fig. 6). Active staining of β-1,3-glucanase on native-PAGE gel was stronger for the DW extract than the K-PO_4 buffer extract (Fig. 6a and b). The active bands showed on native-PAGE gel as G1 and G2 in the DW extract and G1 in the K-PO_4 buffer extract. The active staining of β-1,3-glucanase on the SDS-PAGE gel was stronger in K-PO_4 buffer extract than DW extract (Fig. 6c and d). The active bands showed on SDS-PAGE gel as G3 in DW extract and G3, 85, 64, and 30 kDa in K-PO_4 buffer extract.

Expression patterns of peroxidase in rape (cv. Saturnin) seeds were observed on native-PAGE gels following extraction with DW and 100 mM K-PO_4 buffer at 0, 1, 2, 3, 4, and 5 days after seed germination (Fig. 7). The expression of peroxidase was not observed at the early stage of plant growth. The active staining of peroxidase increased with time during germination. Active staining of peroxidase was stronger earlier in the DW extract than the K-PO_4 buffer extract at 2 days after germination (Fig. 7a and b). The active bands showed as P1 and P2 in both DW and K-PO_4 buffer extract at 5 days after germination.

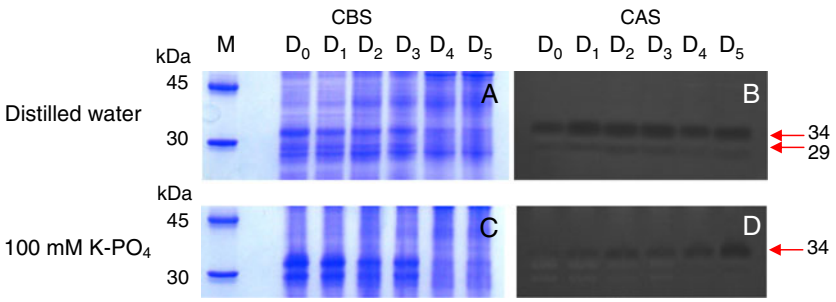

Fig. 5 Chitinase activity staining of rape (*B. napus* L. cv. Saturnin) crude proteins extracted with distilled water (**a** and **b**) and with 100 mM K-PO_4 buffer (**c** and **d**) were stained on SDS-PAGE 12% (w/v) gels with CBS (Coomassie brilliant blue R-250) and CAS (chitinase activity staining by a glycol chitin used as substrate and calcofluor white M2R used for resolution of dark bands). Protein marks (*M*) and days (D_0, D_1, D_2, D_3, D_4, and D_5) after germination

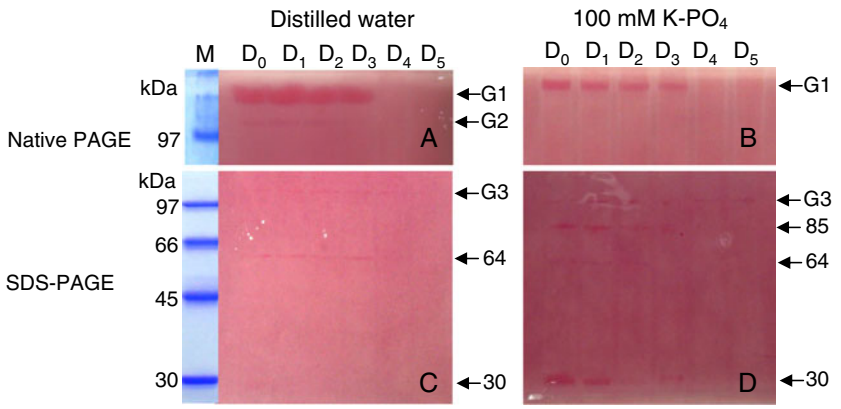

Fig. 6 β-1,3-Glucanase activity staining of rape (*B. napus* L. cv. Saturnin) crude proteins extracted with distilled water (**a** and **c**) and extracted with 100 mM K-PO_4 buffer (**b** and **d**) were stained on native PAGE 10% (w/v) and SDS-PAGE 12% (w/v) gels with 0.15% triphenyltetrazolium chloride solution containing 1 N NaOH. Protein marks (*M*) and days (D_0, D_1, D_2, D_3, D_4, and D_5) after germination

Discussion

Generally, PR proteins are expressed in all plants in response to pathogen infection. The germination of rape (*B. napus*) seeds is enhanced by PR10 protein under abiotic stress such as saline condition (Srivastava et al. 2004). Evaluation of plant chitinases in various seeds including *Benincasa hispida* (Shih et al. 2001), *Secale cereale* (Taira et al. 2001), *Cucumis melo* (Witmer et al. 2003), *Glicine max* (L.) Merr. (Yeboah et al. 1998), *Hordeum vulgare* L. (Swegle et al. 1992), and *Zea mays* L. (Huynh et al. 1992) have already been described.

To investigate an expression pattern of PR proteins in rape seeds during germination, two different extract buffers (DW and K-PO_4) were used to obtain crude enzymes in this study. The protein content was higher in K-PO_4 buffer (pH 7.0) than in DW (pH 6.0) during germination (Fig. 2). In addition, chitinase activity assays revealed that the activity was higher in DW (pH 6.0) extract than in K-PO_4 buffer (pH 7.0) extract (Fig. 3a). The expression pattern of chitinase in rape seeds was evaluated on 10% native-PAGE gel during germination (Fig. 4). Four chitin isozymes (ch1, ch2, 86, and 78 kDa) were primarily expressed in both DW and K-PO_4 buffer extract at 2 days after seeding on a petri dish, and their level of accumulation increased during the germination of Saturnin seeds (Fig. 4b and d). The chitinase from Saturnin seeds was detected in DW extract at 34 and 29 kDa on SDS-PAGE gel, but only at 34 kDa in K-PO_4 buffer extract during germination (Fig. 5a and b). Based on these results, the DW extraction of chitinase from Saturnin seeds was more effective than the K-PO_4 buffer extract. In a study of maize embryos, the expression of 25 kDa chitinase increased during germination and was affected by fungal infection (Cordero et al. 1994).

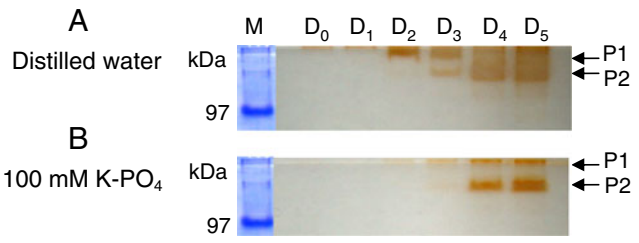

Fig. 7 Peroxidase activity staining of rape (*B. napus* L. cv. Saturnin) crude proteins extracted with distilled water (**a**) and extracted with 100 mM K-PO_4 buffer (**b**) were stained on native PAGE 10% (w/v) gels with 0.46% (v/v) guaiacol and 13 mM H_2O_2 in 50 mM Tris buffer (pH 6.8). Protein marks (*M*) and days (D_0, D_1, D_2, D_3, D_4, and D_5) after germination

Plant chitinase-like proteins from *Panax notoginseng* roots can act as inhibitors of pathogens (Lam and Ng 2001). Several studies have already described an exudation of chitinases from roots. For example, Masuda et al. (2001) reported that cucumber roots produce and secrete a 28 kDa acid chitinase, which is a PR protein, into xylem sap. In addition, Nobrega et al. (2005) demonstrated that chitinase was secreted from the roots of cowpea (*Vigna unguiculata*) seedlings that were grown hydroponically in three different media, including 100 mM sodium acetate buffer (pH 4.5), water (pH 6.0), and 100 mM sodium phosphate buffer (pH 7.5). Santos et al. (2007) demonstrated that the expression of class III chitinase was at about 30 kDa in *Adenanthera pavonina* seedlings. In addition, the presence of an enzyme similar to the isolated thermostable chitinase was detected in exudates released from *A. pavonina* seeds during germination (Santos et al. 2004).

Nobrega et al. (2005) demonstrated that β-1,3-glucanase was secreted from the roots of cowpea (*Vigna unguiculata*) seedlings that were grown hydroponically in three different media, including, 100 mM sodium acetate buffer (pH 4.5), water (pH 6.0), and 100 mM sodium phosphate buffer (pH 7.5). The β-1,3-glucanase in exudates showed high specific activity at low pH in acetate buffer (pH 4.5) and water (pH 6.0). In agreement with these findings, β-1,3-glucanase activity assays conducted in the present study revealed higher activity in DW (pH 6.0) than in K-PO_4 buffer (pH 7.0) (Fig. 3b). Moreover, β-1,3-glucanase active staining

(G1) showed higher activity on the native-PAGE gel following DW (pH 6.0) extraction at 3 days after seeding (Fig. 6a and b). Based on these results, β-1,3-glucanase from rape seed will play an important plant development role as the acid enzyme. The accumulation of β-1,3-Glucanase of 35 kDa detected during germination of maize increased during germination of maize embryos (Cordero et al. 1994).

Peroxidase activity is commonly detected in the growth stage from the early stage of germination to the final step of senescence (Passardi et al. 2005). In addition, Scialabba et al. (2002) demonstrated that peroxidases in the medium surrounding the seed were released during germination of radish (*Raphanus sativus*). Morohashi (2002) also reported that a study of tomato (*Lycopersicon esculentum*) seeds demonstrated that expression of peroxidase genes begins in the early stage of germination. In the present study, peroxidase activity assays revealed higher activity in K-PO_4 buffer (pH 7.0) than in DW (pH 6.0) (Fig. 3c). The P1 and P2 bands showed higher peroxidase activity on native-PAGE gel following DW (pH 6.0) and K-PO_4 buffer (pH 7.0) extraction at 4 and 5 days after seeding (Fig. 7a and b).

Overall, the results of this study will help in the development of strategies for constitutive expression and general utilization of PR proteins involved in plant defense mechanisms.

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References

Antimo DM, Irma T, Lucia S, Lusia F, Michelina R, Giandomenico C, Augusto P, Rosa R (2010) Purification and characterization of a viral chitinase active against plant pathogens and herbivores from transgenic tobacco. J Biotechnol 147:1–6

Asselin A, Grenier J, Cote F (1985) Light-influenced extracellular accumulation of b (pathogenesis-related) proteins in Nicotiana green tissue induced by various chemicals or prolonged floating on water. Can J Bot 63:1276–1283

Bewley J (1997) Seed germination and dormancy. The Plant Cell 9:1055–1066

Boller T (1988) Ethylene and the regulation of antifungal hydrolases in plants. Oxford Sur Plant Mol Cell Biol 5:145–174

Boller T, Gehri A, Mauch F, Vogeli U (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157:22–31

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Caruso C, Chilosi G, Caporale C, Leonardi L, Bertini L, Magro P, Buonocore V (1999) Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum*. Plant Sci 140:87–97

Chance B, Maehly AC (1955) Assay of catalases and peroxidases. Methods in Enzymol 2:764–775

Cook D, Dreyer D, Bonnet D, Howell M, Nony E, Vander-Bosh K (1995) Transient induction of a peroxidase gene in *Medicago truncatula* precedes infection by *Rhizobium melitoti*. Plant Cell 7:43–55

Cordero M, Raventos D, San Segundo B (1994) Differential expression and induction of chitinases and β-1, 3-glucanases in response to fungal infection during germination of maize seeds. Mol Plant-Microbe Inter 7:23–31

Dumas E, Gianinazzi S, Cornu A (1987) Genetic aspects of polyacrylic-acid-induced resistance to tobacco mosaic virus and tobacco necrosis virus in Nicotiana plants. Plant Pathol 36:544–550

Gaudet DA, Laroche A, Frick M, Davoren J, Puchalski B, Ergon A (2000) Expression of plant defence-related (PR-protein) transcripts during hardening and dehardening of winter wheat. Physiol Mol Plant Pathol 57:15–24

Gianinazzi S, Martin C, Vallee J (1970) Hypersensibilite aux virus, temperature et proteines solubles chez le Nicotiana Xanthi nc. Apparition de nouvelles macromolecules lors de la repression de la synthese virale. CR Acad Sci Hebd Seances Acad Sci D 270:2383–2386

Ham KS, Kauffmann S, Albersheim P, Darvill AG (1991) Host-pathogen interactions. XXXIX. A soybean pathogenesis-related protein with β-1, 3-glucanase activity releases phytoalexin elicitor-active heat-stable fragments from fungal walls. Mol Plant-Microbe Inter 4:545–552

Harrison S, Curtis M, McIntyre C, Maclean D, Manners J (1995) Differential expression of Peroxidase isogenes during the early stages of infection of the tropical forage legume *Stylosanthes humilis* by *Collectotrichum gloeosporioides*. Mol Plant Microbe Inter 8:398–406

Huynh Q, Hironaka C, Levine E, Smith C, Borgmeyer J, Shah D (1992) Antifungal proteins from plants. Purification, molecular cloning, and antifungal properties of chitinases from maize seed. J Biol Chem 267:6635

King G, Hussey C, Turner V (1986) A protein induced by NaCl in suspension cultures of *Nicotiana tabacum* accumulates in whole plant roots. Plant Mol Biol 7:441–449

Kobayashi A, Koguchi Y, Kanzaki H, Kajiyama S, Kawazu K (1994) A new type of antimicrobial phenolics produced by plant peroxidase and its possible role in the chemical defense systems against plant pathogens. Z Natur 49:411–414

Lagrimini LM, Burkhart W, Moyer M, Rothstein S (1987) Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. Proc Natl Acad Sci USA 84:7542–7546

Lam SK, Ng TB (2001) Isolation of a small chitinase-like antifungal protein from *Panax notoginseng* (sanchi ginseng) roots. Inter J Biochem Cell Biol 33:287–293

Lingappa Y, Lockwood J (1962) Chitin media for selective isolation and culture of actinomycetes. Phytopathology 52:317–323

Masuda S, Kamada H, Satoh S (2001) Chitinase in cucumber xylem sap. Biosci Biotech Biochem 65:1883–1885

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and β-1, 3-glucanase. Plant Physiol 88:936–942

Meins F, Neuhaus JM, Sperisen C, Ryals J (1992) The primary structure of plant pathogenesis-related glucano-hydrolases and their genes. In: Meins F, Boiler T (eds) Genes Involved in Plant Defense. Springer-Verlag, Vienna, New York, pp 245–282

Monreal J, Reese E (1969) The chitinase of *Serratia marcescens*. Can J Microbiol 15:689–696

Morohashi Y (2002) Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. J Exp Bot 53:1643–1650

Nobrega FM, Santos IS, Da Cunha M, Carvalho AO, Gomes VM (2005) Antimicrobial protein from cowpea root exudates: inhibitory activity against *Fusarium oxysporum* and purification of a chitinase-like protein. Plant Soil 272:223–232

Ornstein L (1964) Disc electrophoresis. I. Background and theory. Ann NY Acad Sci 121:321–349

Pan SQ, Ye XS, Kuc J (1989) Direct detection of β-1,3-glucanase isozymes on polyacrylamide elecrophoresis and isoelectrofocusing gels. Anal Biochem 182:136–140

Park DY, Cho SJ, Shin YC (1986) Changes of protein pattern of mungbean seeds, *Phaseolus aureus* during germination. Kor J Food Sci Technol 18:163–167

Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. Plant Cell Rep 24:255–265

Punja ZK, Zhang YY (1993) Plant chitinases and their roles in resistance to fungal diseases. J Nematol 25:526–540

Quecine MC, Araujo WL, Marcon J, Gai CS, Azevedo JL, Pizzirani-Kleiner AA (2008) Chitinolytic activity of endophytic Streptomyces and potential for biocontrol. Lett Appl Microbiol 47:486–491

Roberts W, Selitrennikoff C (1988) Plant and bacterial chitinases differ in antifungal activity. Microbiology 134:169–176

Santos IS, Da Cunha M, Machado OLT, Gomes VM (2004) A chitinase from *Adenanthera pavonina* L. seeds: purification, characterisation and immunolocalisation. Plant Sci 167:1203–1210

Santos I, Oliveira A, Da Cunha M, Machado O, Neves-Ferreira A, Fernandes K, Carvalh A, Perales J, Gomes V, Cho H (2007) Expression of chitinase in *Adenanthera pavonina* seedlings. Physiol Plant 131:80–88

Scialabba A, Bellani LM, Dell'Aquila A (2002) Effects of ageing on peroxidase activity and localization in radish (*Raphanus sativus* L.) seeds. Eur J Histochem 46:351–358

Sela-Buurlage M, Ponstein A, Bres-Vloemans S, Melchers L, Van den Elzen P, Cornelissen B (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1, 3-glucanases exhibit antifungal activity. Plant Physiol 101:857–863

Shih CY, Khan AA, Jia S, Wu J, Shih DS (2001) Purification, characterization and molecular cloning of a chitinase from the seeds of *Benincasa hispida*. Biosci Biochem 62:501–509

Shinshi H, Mohnen D, Jr Meins F (1987) Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Nati Acad Sci USA 84:89–93

Srivastava S, Fristensky B, Kav N (2004) Constitutive expression of a PR10 protein enhances the germination of *Brassica napus* under saline conditions. Plant Cell Physiol 45:1320–1324

Stintzi A, Heitz T, Prasad V, Wiedemannmerdonoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant "pathogenesisrelated" proteins and their role in defense against pathogens. Biochimie 75:687–706

Swegle M, Kramer K, Muthukrishnan S (1992) Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imbibition. Plant Physiol 99:1009–1014

Taira T, Yamagami T, Aso Y, Ishiguro M, Ishihara M (2001) Localization, accumulation, and antifungal activity of chitinases in rye (*Secale cereale*) seed. Biosci Biotechnol Biochem 65:2710–2718

Trudel J, Asselin A (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal Biochem 178:362–366

van Loon L (1985) Pathogenesis-related proteins. Plant Mol Biol 4:111–116

van Loon L, van Kammen A (1970) Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun' and 'Samsun NN' II. Changes in protein constitution after infection with tobacco mosaic virus. Virology 40:199–211

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

Welinder KG (1992) Plant peroxidases: structure-function relationships. In: Penel C, Gaspar T, Greppin H (eds) Plant peroxidases. University of Geneva, Geneva, Switzerland, pp 1–24

Witmer X, Nonogaki H, Beers EP, Bradford KJ, Welbaum GE (2003) Characterization of chitinase activity and gene expression in muskmelon seeds. Seed Sci Res 13:167–177

Yeboah NA, Arahira M, Nong VH, Zhang D, Kadokura K, Watanabe A, Fukazawa C (1998) A class III acid endochitinase is specifically expressed in the developing seeds of soybean (*Glicine max* (L.) Merr.). Plant Mol Biol 36:407–415

Yedidia I, Benhamou N, Kapulnik Y, Chet I (2000) Induction and accumulation of PR proteins activityduring early stages of root colonizationby the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol Biochem 38:863–873