

Short Communication

## Interaction between Cell Wall and Plasma Membrane via RGD Motif is Implicated in Plant Defense Responses

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**Integrin- and vitronectin-like proteins were found to exist in the pea plasma membrane and cell wall, respectively. The hexapeptide GRGDSP but not GRGESP inhibited either the binding of cell wall protein(s) to plasma membrane protein(s) or the defense response. A possible role of linkage via RGD motif in plant defenses is discussed.**

**Key words:** Cell wall-plasma membrane interaction — Defense response — Extracellular matrix adhesive protein — Integrin — *Pisum sativum* L. — RGD-motif.

In animal cells, an interaction between the plasma membrane and extracellular matrix (ECM) is shown to regulate developmental processes involving changes in polarity, differentiation, division, death and migration (for review, see Hynes 1992). A sequence of RGD conserved in many ECM proteins interacts with integrin in the plasma membrane (Schwartz 1992). Integrin has been demonstrated to function as an apparatus not only for adhesion but also for signaling (Clark and Brugge 1995, Schwartz 1992). Recently, the presence of ECM adhesive proteins and integrin-like proteins has been reported in several plant species (Katembe et al. 1997, Sanders et al. 1991, Schindler et al. 1989, Zhu et al. 1994). In plants, adhesion between plasma membrane and cell wall, pollen tube extension, adaptation to NaCl-stress and perception of gravity are thought to be mediated by the linkage between ECM and integrin-like proteins via RGD-motif conserved in ECM (Wyatt and Carpita 1993). A synthetic hexapeptide, GRGDSP, blocked the aggregation of protoplasts isolated from NaCl-adapted tobacco cells (Zhu et al. 1993), and induced not only abnormal cytoplasmic streaming of mesophyll cells of *Vallisneria* (Ryu et al. 1997) but also abnormal growth of soybean suspension-cultured cells (Schindler et al. 1989). A vitronectin (VN)-like protein with  $M_r$  of 55 kDa was purified from NaCl-adapted tobacco cell (Zhu et al. 1994). However, the role of such a linkage between the cell wall and plasma

membrane in plant defense responses has been obscure. In this report, we first show the importance of RGD-mediated interaction between the cell wall and plasma membrane in the expression of plant defense responses.

Fractions of plasma membranes and cell walls were prepared from etiolated epicotyls of pea (*Pisum sativum* L., cv. Midoriusui) by the methods described by Yoshioka et al. (1990) and Kiba et al. (1995), respectively. Cell wall proteins were collected by solubilizing with 0.5 M NaCl as described previously (Kiba et al. 1995). Plasma membrane proteins (50  $\mu$ g) and cell wall proteins (20  $\mu$ g) were separated by SDS-PAGE on a 4–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan) and then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, U.S.A.). The blots were subjected to western blot analyses with a monoclonal antibody raised against chicken  $\beta$ 1 integrin (Sigma, St. Louis, MO, U.S.A.) and a polyclonal antibody raised against human (H)VN or HVN receptor (R) (Funakoshi, Tokyo, Japan). Cross-reacted proteins were visualized with an alkaline phosphatase-conjugated secondary antibody raised in goat against mouse or rabbit IgG (Funakoshi, Tokyo, Japan) with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP/NBT; Sigma, St. Louis, Mo, U.S.A.).

Biotinylated cell wall proteins were prepared with a protein biotinylation module (Amersham RPN-2202), according to the manufacturer's instruction. Far-western blot analysis was performed by the method of Ye and Baltimore (1994) with slight modifications. The blot of electrophoresed plasma membrane proteins (50  $\mu$ g) was blocked with phosphate buffered saline containing 0.05% Tween 20 (TPBS) with 3% gelatin (Bio-Rad, Hercules, CA, U.S.A.) at 25°C for 1 h, and then incubated with TPBS in the absence or presence of 200  $\mu$ g ml<sup>-1</sup> of the hexapeptide GRGDSP (RGD peptide) or GRGESP (RGE peptide) (Takara, Kyoto, Japan). After incubation at 25°C for 1 h, the membranes were incubated with 100  $\mu$ g ml<sup>-1</sup> of biotinylated cell wall proteins in the absence or presence of RGD or RGE peptide. After incubation at 25°C for 2 h, biotinylated cell wall proteins bound to plasma membrane proteins on the PVDF membrane were visualized with an alkaline phosphatase-conjugated streptavidine and BCIP/NBT.

Abbreviations: ECM, extracellular matrix; (H)VN(R), (human) vitronectin (receptor); PVDF, polyvinylidene difluoride; (T)PBS, phosphate-buffered saline (containing 0.05% Tween 20).

For determination of phytoalexin in pea tissues, excised epicotyl segments (1.5-cm length) from 7-day-old seedlings were divided longitudinally into two parts as described previously (Toyoda et al. 1992). Respective pieces were pretreated with 50  $\mu$ l of RGD or RGE peptide solution at the concentration of 0, 0.03, 0.15, 0.3 and 1.5 mM. After incubation at  $20 \pm 2^\circ\text{C}$  for 0, 1, 3 or 6 h, the pieces were then treated with 50  $\mu$ l of distilled water or the solution of RGD or RGE peptide in the absence or presence of the elicitor (500  $\mu\text{g ml}^{-1}$ ) which was prepared from the pycnospore germination fluid of *Mycosphaerella pinodes* as described previously (Yoshioka et al. 1990). The amount of accumulated pisatin was determined 18 h after the start of elicitor-treatment by HPLC as described previously (Masuda et al. 1983).

To determine the effect of RGD on ATPase activity and superoxide generation, the protein fraction solubilized from the cell wall was preincubated on ice for 6 h in 30 mM Tris-MES (pH 6.5) that contained RGD or RGE peptide at the concentration of 0, 0.03, 0.15, 0.3 or 1.5 mM. The ATPase activity was determined spectrophotometrically by the method of Perlin and Spanswick (1981). About 50 ng of the solubilized cell wall protein was incubated at  $25^\circ\text{C}$  for 20 min in 30 mM Tris-MES (pH 6.5) that contained 3 mM Mg-ATP with or without 100  $\mu\text{g ml}^{-1}$  (glucose equiv.) of the elicitor from *M. pinodes* in the absence or presence of respective concentration of RGD or RGE peptide. The superoxide generation in these fractions was determined by the method described previously (Kiba et al. 1997). The assay mixture was composed of 5  $\mu\text{g}$  of the solubilized cell wall protein, 30 mM Tris-MES (pH 6.5), 20 mM  $\text{MnCl}_2$ , 2.5  $\mu\text{g ml}^{-1}$  of nitroblue tetrazolium (Wako, Osaka, Japan) and 0.5 mM *p*-coumaric acid (Sigma, St. Louis, MO, U.S.A.) in the absence or presence of 100  $\mu\text{g ml}^{-1}$  of the elicitor with or without RGD or RGE peptide. The reaction was started by the addition of 0.5 mM of NADH (Sigma, St. Louis, MO, U.S.A.) and the formation of blue formazan was monitored at 560 nm every 10 seconds for 2 min with a spectrophotometer (Beckman DU series 600; Fullerton, CA, U.S.A.).

As shown in Figure 1, western blot analysis of plasma membrane proteins with the anti-HVNR antibody showed the presence of seven VNR-like proteins with  $M_r$ s of 107, 86, 74, 60, 40, 30 and 27 kDa. The antibody against chicken  $\beta$ 1 integrin also recognized three proteins with  $M_r$ s of 86, 74 and 60 kDa. The 107 and 86 kDa proteins were similar in size to integrin in animal cells (Ruoslahti and Pierschbacher 1987) and to integrin-like proteins in *Arabidopsis thaliana* (Katembe et al. 1997). On the other hand, the 74 kDa protein in pea plasma membrane was similar in size to the soybean protein which was found with anti-HVNR antibody (Schindler et al. 1989). The  $\alpha$  subunit of VNR in animal cells is composed of two disulfide-linked polypeptides, ca. 20 kDa light chain and 120 to 140 kDa

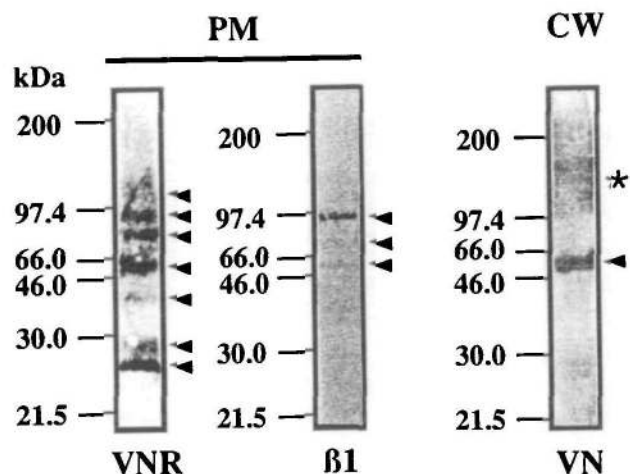
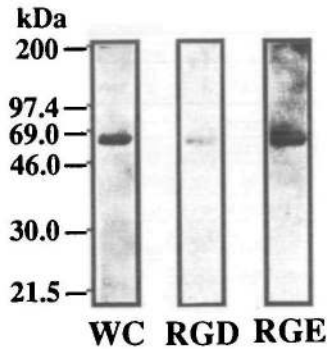


Fig. 1 Western-blot analyses of cell wall proteins and plasma membrane proteins from etiolated pea epicotyls. The cell wall proteins (CW) and plasma membrane proteins (PM) were separated by SDS-PAGE, transferred onto PVDF membranes and then probed with antibodies raised against HVN (VN), HVNR (VNR) and chicken  $\beta$ 1 integrin ( $\beta$ 1). Arrowheads indicate immunoreactive proteins, and the asterisk indicates a broad band of immunoreactive protein. Molecular weights are shown on the left side.

heavy chain (Ruoslahti and Pierschbacher 1987). Therefore, it is probable that the other smaller proteins with  $M_r$ s of 30 and 27 kDa detected by the anti-HVNR antibody in pea plasma membrane are related to light chains of the  $\alpha$  subunit of VNR in animal cells. On the other hand, western-blot analysis of cell wall proteins with an anti-HVN antibody showed at least two positive molecules with  $M_r$ s of 85–180 kDa (a broad band) and 55 kDa (Fig. 1). The 55 kDa protein in the pea cell wall was similar in size to the VN-like 55 kDa protein found in several plant species (Sanders et al. 1991).

The fact that integrin- and vitronectin-like proteins existed in plasma membranes and cell walls, respectively, suggests that an interaction occurs between cell wall protein(s) and plasma membrane protein(s) mediated by RGD-motif. To evaluate such a hypothesis, a far-western blotting analysis was performed. As shown in Figure 2, the biotinylated cell wall protein(s) interacted with a single plasma membrane protein with  $M_r$  of 60 kDa. Furthermore, the binding was markedly inhibited by RGD peptide but not by RGE peptide. These results strongly suggest that cell wall protein(s) directly interacts via RGD motif with a 60 kDa plasma membrane protein which is recognized by both anti-HVNR and anti-chicken  $\beta$ 1 integrin antibodies (Fig. 1).

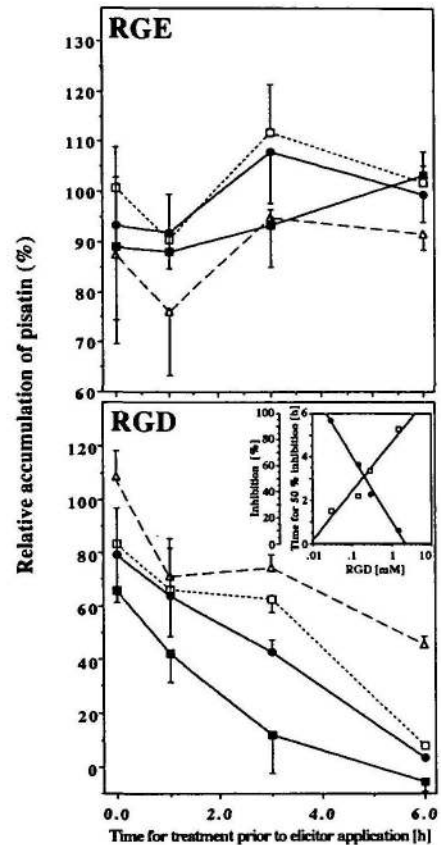
To determine the role of the linkage between cell wall and plasma membrane via RGD-motif in plant defense response, the effect of RGD peptide on the production of the phytoalexin, pisatin, in pea epicotyls was examined. As shown in Figure 3, RGE peptide hardly inhibited the pro-



**Fig. 2** Far-western blot analysis of interaction between cell wall proteins and plasma membrane proteins. The plasma membrane proteins were separated by SDS-PAGE and then electrophoretically transferred onto PVDF membranes. The membrane was incubated at 25°C for 1 h in TPBS in the absence (WC) or presence of RGD and RGE peptides at the concentration of 200  $\mu\text{g ml}^{-1}$ . Then, the membrane was incubated in TPBS that contained 100  $\mu\text{g ml}^{-1}$  of biotinylated cell wall protein in the absence (WC) or presence of RGD and RGE peptides at the concentration of 200  $\mu\text{g ml}^{-1}$ . Molecular weights are shown on the left side.

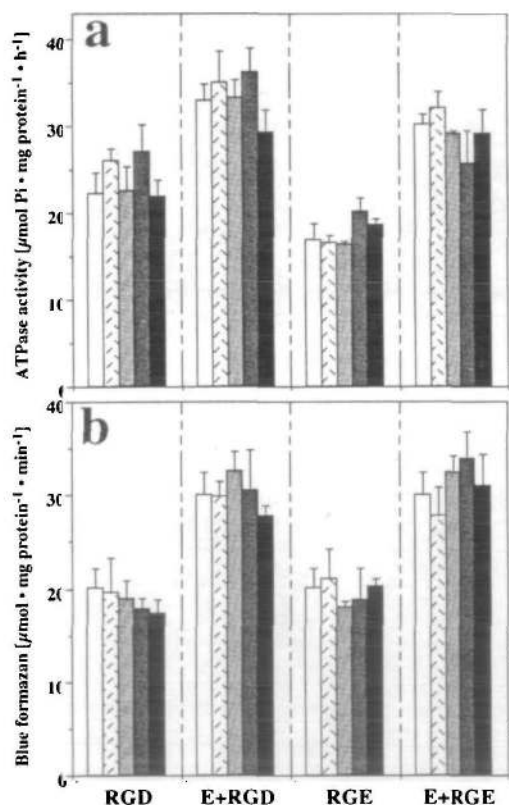
duction of phytoalexin induced by the elicitor. The production of phytoalexin was also scarcely affected by the simultaneous application of 0.03 mM of RGD peptide with the elicitor. Though the application of RGD peptide at more than 0.15 mM tended to inhibit the phytoalexin production in a dose-dependent manner, only 30% of the accumulation was inhibited by 1.5 mM of RGD peptide. On the other hand, the phytoalexin production was severely inhibited to the level of water control by the treatment with RGD peptide at more than 0.15 mM for 6 h prior to elicitor-treatment. Both the inhibition rate of phytoalexin production by RGD peptide when applied for 3 h prior to the elicitor-treatment and the time required for 50% inhibition of the production were expressed as linear equation (Fig. 3, inset). Thus, the inhibitory effect of RGD peptide is correlative to the duration of pretreatment and its concentration. Such a mode of action of RGD peptide on the defense response is quite different from that of the suppressor from *M. pinodes*, that was able to inhibit pea defense responses when applied simultaneously with the elicitor (Yamada et al. 1989, Yoshioka et al. 1992). Moreover, the suppressor was reported to negate the transcriptional activation of a gene for phenylalanine ammonia-lyase, which is a key enzyme in the phytoalexin biosynthetic pathway, even at 15 min after the elicitor-treatment (Wada et al. 1995).

We previously reported that the suppressor from *M. pinodes* inhibited or delayed defense responses in pea plant (for reviews, see Shiraishi et al. 1991, 1994, 1997) and that the suppressor affected cell wall functions such as ATP hydrolyzation and superoxide generation (Kiba et al. 1995, 1996, 1997, Shiraishi et al. 1997). Consequently, the effect



**Fig. 3** Effect of RGD and RGE peptides on the production of a pea phytoalexin, pisatin, with respect to addition time prior to treatment with the elicitor. The epicotyls were pretreated with RGD or RGE peptide at the concentration of 0, 0.03, 0.15, 0.3 and 1.5 mM for 0, 1, 3 or 6 h before the elicitor-treatment. The amount of accumulated pisatin was determined 18 h after treatment with distilled water, 500  $\mu\text{g ml}^{-1}$  of the elicitor from *Mycosphaerella pinodes* and the elicitor plus RGD or RGE peptide at the concentration of 0.03 ( $\Delta$ ), 0.15 ( $\square$ ), 0.3 ( $\bullet$ ) or 1.5 mM ( $\blacksquare$ ), respectively. The relative value was calculated as follows:  $100 \times [(\text{the amount of pisatin in pea epicotyls treated with the elicitor in the presence of respective concentration of RGD or RGE peptide}) - (\text{the amount of pisatin treated with water})] \div [(\text{the amount of pisatin treated with the elicitor alone}) - (\text{the amount of pisatin treated with water})]$ . The amounts of pisatin in respective tissues, which were treated with water and the elicitor for 18 h after preincubation with water for 0, 1, 3 or 6 h, were  $3.8 \pm 0.82 \mu\text{g [g fr wt]}^{-1}$  and  $11.9 \pm 1.55 \mu\text{g [g fr wt]}^{-1}$  (0 h),  $5.9 \pm 0.35 \mu\text{g [g fr wt]}^{-1}$  and  $14.4 \pm 0.23 \mu\text{g [g fr wt]}^{-1}$  (1 h),  $5.4 \pm 0.62 \mu\text{g [g fr wt]}^{-1}$  and  $10.6 \pm 1.54 \mu\text{g [g fr wt]}^{-1}$  (3 h), and  $5.63 \pm 0.51 \mu\text{g [g fr wt]}^{-1}$  and  $15.6 \pm 0.82 \mu\text{g [g fr wt]}^{-1}$  (6 h), respectively. Each value indicates mean and standard deviation (SD) from triplicate experiments. The inset shows the relationship between the concentrations of RGD peptide and the inhibition rate of phytoalexin production by RGD peptide when applied for 3 h prior to the elicitor-application ( $\square$ ) or the time required for 50% inhibition of phytoalexin production by RGD peptide ( $\bullet$ ). The former and the latter correlations were expressed as linear equations of  $Y = 77.763 + 37.887 \times \log X$  ( $R^2 = 0.990$ ) and  $Y = 1.0458 - 3.025 \times \log X$  ( $R^2 = 0.942$ ), respectively.

of RGD peptide on the ATPase activity and superoxide generation in the protein fraction solubilized from pea cell wall was examined. Since the accumulation of phytoalexin in pea tissues was markedly inhibited by RGD peptide when it was applied 6 h prior to the elicitor-treatment, the solubilized protein was pretreated with RGE or RGD peptide for 6 h. The ATPase activity and superoxide generation, however, were not inhibited either by treatment with RGE or RGD peptides. Furthermore, both peptides did not affect the elicitor-enhanced ATPase activity and superoxide generation (Fig. 4a, b). In addition, neither peptide showed any inhibitory effect on the ATPase activity in isolated pea plas-



**Fig. 4** Effect of RGD and RGE peptides on the activities of ATPase (a) and superoxide generation (b) in the protein fraction solubilized from pea cell wall. The solubilized proteins were incubated on ice for 6 h in 30 mM Tris-MES (pH 6.5) that contained RGD or RGE peptide at the concentration of 0 (□), 0.03 (▨), 0.15 (▩), 0.3 (▧) or 1.5 mM (■). a, The activity of ATPase was determined at 25°C for 20 min in 30 mM Tris-MES (pH 6.5) that contained 3 mM Mg-ATP with or without 100 µg ml<sup>-1</sup> (glucose equiv.) of the elicitor from *M. pinodes* in the absence or presence of RGD or RGE peptide at the respective concentration as described above. b, Superoxide anion was measured in 30 mM Tris-MES (pH 6.5) that contained 0.5 mM NADH, 20 mM MnCl<sub>2</sub>, 0.5 mM *p*-coumaric acid and 2.5 µg ml<sup>-1</sup> of nitroblue tetrazolium with or without 100 µg ml<sup>-1</sup> of fungal elicitor in the absence or presence of RGD and RGE peptides at the respective concentration as described above. Each value represents the mean with standard deviation (SD) of results from triplicate experiments.

ma membranes (data not shown). Thus, the RGD peptide did not inhibit directly either the cell wall or plasma membrane function indicating that the action of RGD peptide is different from that of the *M. pinodes*-suppressor (Kato et al. 1993, Kiba et al. 1995, 1996, Shiraishi et al. 1991, 1994, 1997, Yoshioka et al. 1990).

Our previous reports suggested that the plant cell wall plays crucial roles in the recognition of pathogens and in determination of plant-pathogen specificity (Kiba et al. 1995, 1996, 1997, Shiraishi et al. 1997, 1998). However, the mechanism of signal transmission from the cell wall to plasma membrane during defense responses has been obscure for a long time. The results of the present study are summarized as follows: (1) VN-like and VNR-like proteins were found to exist in the pea cell wall and plasma membrane, respectively, and, the adhesion of both proteins is blocked by RGD peptide but not by RGE peptide; (2) the pea defense response is severely inhibited by RGD peptide when applied prior to the elicitor-treatment but not by RGE peptide; and, (3) RGD peptide does not affect directly the ATPase and superoxide generation in pea cell wall. These findings lead us to a concept that the RGD peptide may not inhibit directly the enzymes involved in the signal transmission pathway leading to defense responses unlike the suppressor from *M. pinodes*. The RGD peptide rather may separate a constitutive linkage between cell wall and plasma membranes dependent on VN- and VNR-like proteins via RGD motif, resulting in interception of signal transmission from the cell surface into the cells, followed by inhibition of the defense response. In other words, fungal signals are recognized on the plant cell wall and the putative second signal(s) may be transmitted into intracellular space by the interaction between VN-like and integrin-like protein(s) via RGD motif. Further characterization of proteins, which are involved in such a linkage, is underway.

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