Characterization of the *in vitro* Activities of the P1 and Helper Component Proteases of *Soybean mosaic virus* Strain G2 and *Tobacco vein mottling virus*

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(Received on February 28, 2012; Revised on April 30, 2012; Accepted on April 30, 2012)

Potyviruses express their RNA genomes through the production of polyproteins that are processed in host cells by three virus-encoded proteases. Soybean plants produce large amounts of protease inhibitors during seed development and in response to wounding that could affect the activities of these proteases. The *in vitro* activities of two of the proteases of *Soybean mosaic virus* (SMV) and *Tobacco vein mottling virus* (TVMV) were compared in the rabbit reticulocyte lysate *in vitro* translation system using synthetic RNA transcripts. Transcripts produced from SMV and TVMV cDNAs that included the P1 and helper component-protease (HC-Pro) coding regions directed synthesis of protein products that were only partially processed. Unprocessed polyproteins were not detected from transcripts that included all of the P1, HC-Pro, P3 and portions of the cylindrical inclusion protein coding regions of either virus. Addition of soybean trypsin inhibitor to *in vitro* translation reactions increased the accumulation of the unprocessed polyprotein from TVMV transcripts, but did not alter the patterns of proteins produced from SMV. These experiments suggest that SMV- and TVMV-encoded proteases are differentially sensitive to protease inhibitors.

Keywords : Helper-component protease, *in vitro* translation, P1 protease, trypsin inhibitor

Viruses in the family *Potyviridae*, the largest family of plant viruses (Adams et al., 2012), cause serious economic losses in numerous food and ornamental crops. Potyviruses have single-stranded positive-sense RNA genomes of approxi-

mately 10 kb and produce one large polyprotein of approximately 350 kDa that is proteolytically processed to form individual functional proteins (Adams et al., 2012). The carboxyl-terminal two thirds of potyvirus polyproteins are cleaved by a virus-encoded protease, designated nuclear inclusion a (NIa) (Carrington and Dougherty, 1987), which functions similarly to the 3C proteases of animal picornaviruses (Adams et al., 2005). Potyviruses express two other proteases, P1 and helper component protease (HC-Pro), which catalyze cleavage at their own carboxyl-termini. P1 protease is a serine protease, and HC-Pro is classified as a papain-like cysteine protease (Adams et al., 2005). The Gly-Gly cleavage sites at the carboxyl-termini of HC-Pros are conserved among potyviruses. The cleavage sites at the carboxy-termini of P1 protease are less well conserved, but cleavages occur most commonly between Phe-Ser and Try-Ser dipeptides (Adams et al., 2005).

Soybean mosaic virus (SMV) and *Tobacco vein mottling virus* (TVMV) are both members of the family *Potyviridae* and have relatively narrow host ranges. SMV infects mainly soybean (*Glycine max* (L.) Merr.) and has been shown experimentally to infect at least 10 additional plant species, mostly in the family *Fabaceae* (Hill, 1999). In addition to tobacco (*Nicotiana tabacum* L.), TVMV has been shown to infect eight other plant species, mostly in the family *Solanaceae* (Sun et al., 1974).

Soybean seeds contain high levels of two classes of protease inhibitors, soybean trypsin inhibitor and Bowman-Birk inhibitor, both of which inhibit serine proteases, and can represent up to 6% of the mass of mature soybean seeds (Laskowski and Kato, 1980). The soybean trypsin inhibitor gene is expressed early in soybean seed development (Walling et al., 1986). After germination, the levels of both classes of protease inhibitors decline during seedling growth and are only detected in cotyledons of mature plants (Tan-

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Wilson et al., 1985). In mature soybean plants, protease inhibitors can be induced in response to insect predation (Koiwa et al., 1997). In addition to soybean trypsin inhibitor, soybean plants induce cysteine protease inhibitors, phyto-cystatins, in response to wounding that inhibit papain-like digestive enzymes (Botella et al., 1996).

In these experiments, we tested the hypothesis that protease inhibitors that are expressed during soybean seed development and persist during germination and early seedling development differentially affect the activities of potyvirus-encoded proteases. This hypothesis was based on observations that SMV, as a seed-transmitted virus (Hill, 1999), must function in the tissues of young germinating seedlings that contain high levels of protease inhibitors, and that the P1 protease of TVMV cleaves autocatalytically in the wheat germ *in vitro* translation system, but not in the rabbit reticulocyte system, which was attributed to the presence of protease inhibitor(s) in rabbit reticulocyte lysates (Gopinath and Rhoads, 1991).

To test the hypothesis, we compared *in vitro* translation products synthesized in the rabbit reticulocyte lysate translation system programmed with *in vitro* transcripts from SMV or TVMV cDNA templates. *In vitro* transcripts were synthesized from restriction enzyme-cleaved full-length cDNA clones of SMV (pALE53, kindly provided by Dr. John Hill, Iowa State Univ.) and TVMV (pXBSIN) (Domier et al., 1989) that had been cleaved with *Bam*HI at nucleotide positions 2319 and 1933, respectively. Transcripts were synthesized using bacteriophage T7 RNA polymerase and 500 ng linearized plasmid DNA templates as recommended by the manufacturer (Promega, Madison, WI, USA). The transcripts included P1 and HC-Pro coding regions of SMV and TVMV (Fig. 1, A and B) and were translated in the rabbit reticulocyte lysate *in vitro* translation system (Promega) in the presence of S³⁵ methionine (Dupont, Boston, MA, USA) as recommended by the manufacturer. Radioactively labeled proteins were analyzed on 8% polyacrylamide SDS gels. Protein bands were detected by exposing dried gels to X-ray film (Biomax, Kodak, Rochester, NY, USA) for 12–24 hr. The *Bam*HI transcripts of SMV produced protein bands of about 35, 43 and 80 kDa on polyacrylamide gels. The 80-kDa protein band corresponded to the expected size of the unprocessed product (Fig. 1C, lane 2). The 35- and 43-kDa products may have been derived from proteolytic processing of the 80-kDa protein and could represent P1 and HC-Pro, respectively. A strong band of about 70 kDa appeared in the *in vitro* translations of the *Bam*HI transcripts of TVMV corresponding to the expected size of the unprocessed translation product (Fig. 1C, lane 3). In addition, lesser amounts of proteins of approximately 38, 50, 60, and 84 kDa were observed (Fig. 1C, lane 3). Hence, neither of the amino terminal poly-

Fig. 1. Schematic representation of the possible protein products of *Bam*HI transcripts of *Soybean mosaic virus* (SMV, A) and *Tobacco vein mottling virus* (TVMV, B) in rabbit reticulocyte lysate *in vitro* translation system. Molecular masses of SMV and TVMV proteins were predicted from amino acid sequences. *In vitro* transcripts were synthesized from cDNA templates and translated in a rabbit reticulocyte lysate *in vitro* translation system. Radioactively labeled proteins were analyzed on 8% polyacrylamide SDS gels. (C) Lane 1: no-template control, 2: *Bam*HI transcripts of SMV (arrows indicate proteins of 35, 43 and 73 kDa), 3: *Bam*HI transcripts of TVMV (arrow indicates protein of 70 kDa). Bars at right represent migrations of pre-stained protein molecular mass standards.

protein fragments of SMV or TVMV was completely processed in the rabbit reticulocyte lysate system, but more of the TVMV translation products remained unprocessed than those of SMV.

A second set of *in vitro* translation reactions was performed with SMV and TMV transcripts that included P1, HC-Pro, P3 and portions of the cylindrical inclusion (CI) protein coding regions (Fig. 2, A and B) by cleaving the SMV and TVMV cDNA templates with *Dra*I (position 5386) and *Xba*I (position 3544), respectively. Translation reactions with *Dra*I transcripts of SMV produced major protein bands of 30, 35, 43, 68 and 80 kDa (Fig. 2C, lane 2). The 35-kDa and 43-kDa protein bands had relative mobilities very similar to the proteins produced from the

Fig. 2. Schematic representation of the possible products from *DraI* transcripts of *Soybean mosaic virus* (SMV, A) and *XbaI* transcripts of *Tobacco vein mottling virus* (TVMV, B) in rabbit reticulocyte lysate *in vitro* translation system. The molecular masses of SMV and TVMV proteins were predicted from amino acid sequences. *In vitro* transcripts were synthesized from cDNA templates and translated in a rabbit reticulocyte lysate *in vitro* translation system. Radioactively labeled proteins were analyzed on 8% polyacrylamide SDS gels. (C) Translation products synthesized from *DraI* transcripts of SMV: lane 1: no-template control, 2: *DraI* transcripts. Arrows indicate major translation products of about 30, 35, 68, and 80 kDa. (D) Translation products synthesized from *XbaI* transcripts of TVMV: lane 1: no-template control, 2: *XbaI* transcripts. Arrows indicate major translation products of about 43 and 80 kDa. Bars at right represent migrations of pre-stained protein molecular mass standards.

translation of *BamHI* transcripts (Fig. 1C, lane 2) and likely represented the same proteins. A protein band of about 95 kDa, the expected size of the P3-CI polypeptide, was not detected (Fig. 2C, lane 2). Translation reactions with the *XbaI* transcripts of TVMV produced large amounts of an 80-kDa protein, which could have resulted from cleavage between the HC-Pro and P3, but not between P1 and HC-Pro (Fig. 2D, lane 2). In contrast, *BamHI* and *DraI* transcripts of SMV predominately produced protein bands that corresponded to the expected masses of the processed forms of P1 and HC-Pro. The 42-kDa product, which is the expected mass of the P3 protein, was not observed in translations of TVMV *BamHI* transcripts (Fig. 2D, lane 2).

The first two studies showed that the SMV and TVMV

P1 proteases functioned poorly in the rabbit reticulocyte translation system when expressed from *in vitro* transcripts. Even so, the SMV P1 protease appeared to be somewhat more active than that produced by TVMV. These results for P1 protease activities are similar to those reported for TVMV and *Tobacco etch virus* (TEV) (Gopinath and Rhoads, 1991; Verchot et al., 1991). The reduced activity of P1 proteases in the rabbit reticulocyte lysate could result from either the presence of protease inhibitors or the absence of a factor required for full P1 protease activity.

Furthermore, we directly tested the effects on protease cleavage by P1 and HC-Pros of the two viruses by the addition of soybean trypsin inhibitor to the *in vitro* translation reactions. Soybean seeds contain about 1.2 mg/g of soybean trypsin inhibitor (Kim et al., 1985). To test the effects of the protease inhibitor on SMV and TVMV P1 and HC-Pro protease activities, soybean trypsin inhibitor (Sigma, St. Louis, MO, USA) was added to the rabbit reticulocyte lysate reactions to final concentrations of 0.2, 0.5, 1.0, and 1.2 mg/ml. Then the *DraI* transcripts of SMV and *XbaI* transcripts of TVMV were added individually to reactions and incubated as described above. The *DraI* transcripts of SMV produced the same series of translation products with and without soybean trypsin inhibitor (data not shown). In contrast, the *XbaI* transcripts of TVMV produced a new protein band of about 120-kDa beginning at a concentration of 0.5 mg/ml soybean trypsin inhibitor (Fig. 3). This 120-kDa protein corresponded to the expected mass for the unprocessed protein produced by TVMV *XbaI* transcripts. The observation that the pattern of *in vitro* translation products produced by TVMV P1 and HC-Pro was altered by the addition of soybean trypsin inhibitor, but not that produced by SMV supports the hypothesis that the proteases of SMV and TVMV are differentially sensitive to protease inhibitors. Because P1 and HC-Pro are required for multiple functions in the virus life cycle (Plisson et al., 2003), tissue-specific loss of activity of one or more proteases could limit the spread of the virus into or out of those tissues.

In related studies, García et al. (1993) and Wen et al.

Fig. 3. *In vitro* processing of *XbaI* transcripts of *Tobacco vein mottling virus* (TVMV) with soybean trypsin inhibitor. Lane 1: no-template control, lanes 2–6: *XbaI* transcripts of TVMV with soybean trypsin inhibitor added to final concentrations of 0.0, 0.2, 0.5, 1.0, and 1.2 mg/ml, respectively. Arrow indicates 120 kDa protein band.

(2004) showed that cystatins, inhibitors of papain-like cysteine proteases, slightly inhibited the activity of *Plum pox virus* (PPV) HC-Pro, but had very little or no effect on the activity of PPV NIa protease. The TEV NIa protease also was resistant to inhibition by a wide range of protease inhibitors (Dougherty et al., 1989). Similar to the results reported here for soybean trypsin inhibitor and TVMV, high concentrations of cystatins were required to produce low levels of inhibition of PPV HC-Pro, limiting the usefulness of the selected protease inhibitors as antiviral agents. The apparent inhibition of TVMV HC-Pro by soybean trypsin inhibitor is surprising because potyviral HC-Pros have been described as true cysteine proteases that are related to papain (Maia et al., 1996) and soybean trypsin inhibitor typically does not inhibit the activities of papain-like cysteine proteases (Yamamoto and Ikenaka, 1967). It is also possible that the activity of TVMV HC-Pro was more sensitive than SMV HC-Pro to the addition of high levels of exogenous proteins to the *in vitro* translation reactions.

Protease inhibitors have proven to be valuable tools in managing some of the most recalcitrant human virus diseases (De Clercq, 2004). The often successful management of *Human immunodeficiency virus* infections with a combination of protease inhibitors is especially noteworthy (Ray et al., 2010). Protease inhibitors also have shown promise for the treatment of chronic *Hepatitis C virus* infections (Kronenberger and Zeuzem, 2009). The work with human diseases illustrates that the selection of a highly effective protease inhibitor with low toxicity is essential for successful antiviral treatments. Hence, it may be possible to use protease inhibitors to provide resistance to viruses in plants through the careful evaluation and selection of highly effective protease inhibitors.

Acknowledgement

This work was supported by a grant from the Next-Generation Biogreen 21 Program (PJ008603), Rural Development Administration, Republic of Korea.

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