From enzyme to zymogen: engineering Vip2, an ADP-ribosyltransferase from Bacillus cereus, for conditional toxicity

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The adenosine diphosphate (ADP)-ribosyltransferase, Vip2 (vegetative insecticidal protein), from Bacillus cereus in combination with another protein from the same organism, Vip1, has insecticidal activity against western corn rootworm larvae. The Vip2 protein exerts its intracellular poisoning effect by modifying actin and preventing actin polymerization. Due to the nature of this toxin, expression of Vip2 in planta is lethal. In this work, we attempted to build an enzyme precursor (proenzyme,zymogen) that would silently reside in one biological system (e.g. plants or yeast) and be activated in the other (insect larvae). Our approach involved engineering a random propeptide library at the C-terminal end of Vip2 and selecting for malfunctional enzyme variants in yeast. A selected proenzyme (proVip2) possesses reduced enzymatic activity as compared with the wild-type Vip2 protein, but remains a potent toxin toward rootworm larvae. In addition, upon analysis of the digestive fate of the engineered enzyme precursor in rootworm larvae, we demonstrated that ‘zymogenized’ Vip2 can be proteolytically activated by rootworm digestive enzyme machinery. This report represents an example of applying a protein engineering strategy for the creation of a plant-tolerated, zymogen-like form of an otherwise toxic protein. This approach may outline a novel path to address challenges associated with utilizing toxic proteins in certain biotechnological applications.

Keywords: ADP-ribosyltransferase/conditional toxicity/proenzyme/Vip2/zymogen

Introduction

Vip2 is a vegetative insecticidal protein (Vip) produced by the spore-forming bacterium Bacillus cereus (B. cereus) during the vegetative phase of growth (Warren et al., 1996). This protein, in combination with another protein from the same organism (Vip1), is toxic to western corn rootworm (WCRW) larvae. Neither Vip1 nor Vip2 protein is an effective toxin against rootworm on its own, however (Warren, 1997). WCRW is a major pest of corn in the United States, causing the most substantial plant damage by feeding exclusively on corn roots (Branson and Ortman, 1970). It is assumed, that the Vip1–Vip2 system belongs to a family of binary bacterial toxins resembling mammalian clostridial toxins of the C2 (Aktories et al., 1986) and iota-like family (Stiles and Wilkins, 1986). These toxins are composed of two independent polypeptide chains that work synergistically; one protein component binds a cell surface receptor and facilitates the delivery of the second component, the toxic enzymatic moiety, into target cells. Vip1, the binding part of the binary system, presumably targets a putative receptor in the WCRW gut membrane and provides a pathway for enzymatically active Vip2 to enter into cells. Vip2 is an ADP-ribosyltransferase that exerts an intracellular poisoning effect by modifying actin and thus preventing actin polymerization and microfilament network formation (Han et al., 1999).

In a broader sense, Vip2 is a representative of the family of ADP-ribosyltransferases performing their key biological functions via protein modification. In this complex enzymatic reaction, ADP-ribosyltransferase catalyzes transfer of the ADP-ribose group of nicotinamide adenine dinucleotide (NAD) to a target protein with nicotinamide release (Rappuoli and Pizza, 1991). In a more refined sense, Vip2 belongs to a family of actin-ADP-ribosylating toxins together with Clostridium botulinum C2 toxin (Aktories et al., 1986), Clostridium perfringens iota toxin (Vandekerckhove et al., 1987), Clostridium spiroforme toxin (Popoff and Boquet, 1988) and an ADP-ribosyltransferase produced by Clostridium difficile (Popoff et al., 1988). Covalently modified actin disturbs the cellular equilibrium between monomeric and polymeric actin and contributes to the cytopathology of the toxins (Aktories and Wagner, 1992).

Examination of the binary Vip1–Vip2 system in planta has been hampered by the fact that expression of Vip2 in corn results in serious developmental pathology and phenotypic alterations. These observations suggested that Vip2 expression could be a useful system to explore engineering strategies for manipulation of toxin effects. In this case we wished to address the following questions: Would it be possible to engineer a Vip2-ADP-ribosyltransferase for conditional toxicity? What modifications can be implemented in this protein to make it benign in one living system (e.g. corn) and toxic in another (e.g. insect larvae)? Could we design a system that would enable efficient selection for functionally impaired Vip2 variants?

In this report we have designed a simple in vivo system for selection of malfunctional Vip2 proteins and outlined a protein engineering approach as a model strategy on how to cope with this complex problem. These data suggest potential steps toward manipulation of other toxins which may possess undesirable characteristics in planta or other biological systems.

Materials and methods

Microbial strains, plasmids and expression constructs

During the course of this work, Escherichia coli (E. coli) strain DH5α has been used for routine cloning experiments. Proteins were expressed in E. coli strain BL21-Gold (DE3) purchased...
from Stratagene (La Jolla, CA, USA). For yeast transformation, a strain of Saccharomyces cerevisiae (S. cerevisiae) INVSc1 from Invitrogen (Carlsbad, CA, USA) has been used.

Two commercially available yeast expression vectors, the high-copy number pYES2 (Invitrogen, Carlsbad, CA, USA) and a low-copy number p416GALS (ATCC, Manassas, VA, USA), have been used for inducible protein expression in S. cerevisiae. These plasmids are shuttle vectors and can be propagated both in E. coli and S. cerevisiae.

A synthetic, maize optimized vip2 gene (Warren et al., 2000) coding for the mature form of Vip2 protein was introduced into the yeast expression vector pYES2 with a BamHI-EcoRI cassette, producing the plasmid pMJ1. In addition, during subcloning from the original source vector, two other genetic elements located downstream of the Vip2 gene, inverted intron #9 from maize phosphoenolpyruvate carboxylase gene (Hudspeath and Grula, 1989) and a 35S transcription terminator from cauliflower mosaic virus (Pietrzak et al., 1986) comprising nucleotides 7562–7631 on the virus sequence map (Franck et al., 1980) were included in the subcloned BamHI-EcoRI fragment.

It is believed that the mature secreted form of Vip2 protein from B. cereus starts with amino acid Leu54 (Warren et al., 2004). We have followed that in this work by expressing a construct which retains this exact sequence. In order to attach propeptide sequences to the Vip2 protein, a unique AatII site was engineered at the end of vip2 gene (in pMJ1) by replacing the last codon AAC (Asn) with TCC (Ser). Since the last amino acid substitution (N462S) does not affect Vip2 toxicity in yeast, we continue to refer to this protein/gene variant as a ‘wt’. A high-copy yeast expression plasmid carrying ‘wt’ vip2 gene in pYES2 backbone was designated pMJ5 and a p416GALS-based low-copy number version with ‘wt’ vip2 gene became pMJ7. Plasmid pMJ7 was prepared by subcloning BamHI-XhoI cassette carrying ‘wt’ Vip2 gene from pMJ5 into p416GALS.

For protein production in E. coli, expression constructs in the pET29a system (Novagen, Madison, WI, USA) were prepared. pMJ23 expression plasmid has ‘wt’ vip2 gene (from pMJ5) inserted in pET29a via SacI-XhoI sites, providing expression of Vip2 protein with a N-terminally attached S-tag. Plasmid construct expressing the S-tag version of Vip2 protein with a N-terminally attached S-tag. Plasmid construct expressing the S-tag version with ‘wt’ Vip2 gene in pYES2 backbone was designated pMJ5 and a p416GALS-based low-copy number version with ‘wt’ vip2 gene became pMJ7. Plasmid pMJ7 was prepared by subcloning BamHI-XhoI cassette carrying ‘wt’ Vip2 gene from pMJ5 into p416GALS.

Preparation of a propeptide library by random elongation mutagenesis

Randomized codons were incorporated into a synthetic oligonucleotide that was used as a forward primer for PCR amplification of the region localized downstream of the vip2 gene. An NNS triplet was used for complete codon randomization, where N represents equal amount (25%) of each nucleotide and S is 50% each G and C. The reverse oligonucleotide initiated deoxyribonucleic acid (DNA) synthesis from the plasmid backbone. In the first round of mutagenesis, a stretch of 21 codons were completely randomized. Our strategy to generate the proenzyme molecule (proVip2) rested on preserving amino acids deemed critical to survive in yeast as determined during initial selection. In the second round of mutagenesis, seven out of 21 amino acids preselected in the first round of mutagenesis were then randomized. The following synthetic oligonucleotides were used for randomizing of seven positions: 5’-GATCAGGACGTCCGT AGATGGGTA(NNS)GGTGAAATTC(NNS)2TGGGT ACATGGAGATGG(NNS)2TAGATCTGTTGTACACAAA GTGGAGTAG-3’ (forward primer) and 5’-GACGCAGCAGAAAACCTTCTCAAG-3’ (reverse primer). The amplified piece of DNA was digested by AatII + MluI and inserted into pMJ7 backbone digested with the same restriction enzymes.

Selection for functional propeptides in yeast

The propeptide library prepared in pMJ7 plasmid was transformed into S. cerevisiae INVSc1 using EZ Yeast Transformation Kit from Zymo Research (Orange, CA, USA). Yeast survivors were selected under condition of leaky expression on synthetic defined media lacking uracil plates supplemented with 4% raffinose. The presence of raffinose as a carbon source in media does not induce or repress transcription from galactose (GAL) promoter. yeast minimal synthetic defined media and -ura dropout supplement were purchased from Clontech (Palo Alto, CA, USA).

Expression of vip2 variants and preparation of protein extracts

Proteins were expressed in E.coli BL21-Gold (DE3) cells. Hundred milliliter of Luria-Bertani media supplemented with kanamycin (50 μg/ml) were inoculated with 1 ml of overnight culture and grown for 3 h (OD600 = 0.5–0.8) at 37°C before induction with 1 mM IPTG and grown for another 3.5 h. Cells were collected by centrifugation and resuspended in 2 ml of 50 mM Tris–HCl, pH7.2, 50 mM NaCl. The cell suspension was lysed by use of the French press (Thermo Electron Corporation, Waltham, MA, USA) and soluble proteins were recovered following centrifugation at 13 000g for 15 min at 4°C. For subsequent ADP-ribosylation assays, preliminary dot blot and western blot analyses were used to arrive at approximately equivalent dilutions of Vip2 and proVip2.

ADP-ribosylation assay

An in vitro ADP-ribosylation assay was carried out at 37°C in a medium containing 10 mM Tris–HCl, pH7.5, 1 mM CaCl2, 0.5 mM ATP, 0.25 μM [32P] NAD, 1 μg non-muscle actin (Cytoskeleton, Inc., Denver, CO, USA) and 2.5 ng of enzyme in a total volume of 25 μl. The enzymatic reaction was stopped by adding SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) sample buffer and boiling for 3 min. One half of the reaction volume was subjected to SDS–PAGE, blotted onto 0.2 μm polyvinylidene fluoride (PVDF) membrane (Invitrogen, Carlsbad, CA, USA) and processed by autoradiography.
**Protein extraction from corn root**

Two hundred and fifty milligrams of corn root material was homogenized in 200 μl of 50 mM sodium carbonate buffer, pH8.0 supplemented with 10 mM ethylenediaminetetraacetic acid, 0.05% Tween 20, 0.05% Triton X-100, 100 mM NaCl, 1 mM AEBSF, 1 mM leupeptin and 1× Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). After homogenization, soluble protein extract was recovered by centrifugation at 12,000g for 15 min. Ten microliters of root extract was used for the ADP-ribosylation assay.

**Digestive fate of proteins in WCRW larvae**

In order to explore the fate of proteins in the WCRW digestive system, we used a feeding assay, in which rootworm larvae were fed either Vip2 or its engineered zymogenic form, proVip2, an artificial diet according to the method of Walters et al. (2008). Concentrated protein extract from 10 ml of E. coli BL21(DE3) cell culture expressing vip2 variant was incorporated into the diet. For Vip2 protein detection in whole body homogenates, 60 rootworm larvae were fed on artificial diet containing Vip2 protein (or its engineered variant) for 30 or 90 min. After feeding, larvae were transferred into 1.5 ml Eppendorf tubes and stored at –80°C until further processing. Larvae were homogenized in SDS–PAGE sample buffer containing 2× Complete Protease inhibitor cocktail (Roche Diagnostics) and heated to 100°C for 5 min. After centrifugation, extracts from homogenized rootworm larvae were separated by SDS–PAGE and blotted onto PVDF membrane. Vip2 proteins were detected with rabbit anti-Vip2 antibody and visualized by horse radish peroxidase-labeled protein A using SuperSignal West Dura chemiluminiscent substrate (Pierce, Rockford, IL, USA) or by alkaline phosphatase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) followed by nitroblue tetrazolium/5-bromo-4-chloro-indolyl-phosphate (NBT/BCIP) detection (Pierce).

For Vip2 protein detection in frass (=feces), rootworm larvae were fed on artificial diet with incorporated Vip2 protein variant for a period of 3 days, before excrement material was collected into 200 μl of enzyme assay buffer containing 10 mM Tris–HCl, pH7.5, 1 mM CaCl₂, 0.5 mM ATP. Collected soluble frass material was analyzed for the presence of enzymatic activity using the ADP-ribosylation assay described earlier and also examined by western blot to assess proteolytic processing. Vip2 antigen was detected with rabbit anti-Vip2 antibody and visualized by Alkaline Phosphatase-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) followed by NBT/BCIP detection (Pierce).

**Plant transformation**

Maize transformation was performed at the Syngenta Biotechnology, Inc., transformation facility (Research Triangle Park, NC, USA) with the method described by Negrotto et al. (2000). The vector for Agrobacterium-mediated maize transformation contains the phosphomannose isomerase (PMI) gene for selection of transgenic lines (Negrotto et al., 2000). The expression cassette comprises, in addition to the proVip2 gene, the maize metallothionein-like (MT-L) gene promoter (De Framond, 2004), extra-cytoplasmic (apoplast) targeting peptide from maize pathogenic-related protein (Casanova et al., 1991) and 35S transcription terminator (Pietrzak et al., 1986).

**Results and discussion**

**A propeptide concept**

Bacterial ADP-ribosylating toxins are proteins produced by pathogenic bacteria, which are usually secreted into the extracellular medium and cause disease by altering the metabolism of eukaryotic cells (Rappuoli and Pizza, 1991; Holbourn et al., 2006). These enzymes catalyze the transfer of the ADP-ribose group from NAD to a target protein with nicotinamide release. Since actin, the major cytoskeleton forming protein in eukaryotic cells, is the primary ribosylation target for Vip2-ADP-ribosyltransferase, the intracellular expression of Vip2 in plant cells could be a real challenge. Our early maize transformation experiments with Vip2 indicated that all transgenic plants had an aberrant phenotype and problems in development. Growth of transformed plants ceased at the very early developmental stage.

In Nature, many proteins with cytotoxic enzymatic function (e.g. proteases) are synthesized in the form of inactive precursors (zymogens, proenzymes – Lazure, 2002). After reaching the proper environment, these precursors are often processed into functional enzymes by other proteases or in an autocatalytic manner (McQueney et al., 1997).

In this report we wanted to implement a similar strategy for repairing Vip2 toxicity in plants and engineer a ‘zymogenized’ form of VIP2 toxin (Vip2 proenzyme, proVip2). We presumed that proper extension of the polypeptide chain of Vip2-ADP-ribosyltransferase could interfere with its enzymatic function by four potential mechanisms: (i) steric blocking of the active site, (ii) interference with the NAD-binding site, (iii) imparting a change in enzyme conformation, or (iv) introducing a decrease in overall protein stability. Since the C-terminal end of Vip2 is in closer proximity to the functional sites of the protein than the N-terminus (Fig. 1), we envisioned that extension of the C-terminal part of the protein might have a better chance to mask Vip2 enzymatic activity. In order to find functional propeptide sequences, we had to design a genetic system that would efficiently select for Vip2 variants with suppressed enzymatic function.

**Yeast: a viable tool for selection of malfunctional Vip2 proteins**

Vip2 belongs to the family of actin-ADP-ribosylating toxins. This NAD-dependent enzyme modifies monomeric actin at Arg177 to block polymerization, leading to loss of cytoskeleton and cell death (Han et al., 1999). Since actin is one of the most conserved proteins throughout the various species including mammalian, yeast and higher plants (Goodson and Hawse, 2002), we presumed that expression of the Vip2-ADP-ribosyltransferase in a model yeast organism, S. cerevisiae, would be lethal. In that case, we would be able to transform yeast cells with a library of mutagenized/engineered Vip2 genes and select for defective Vip2 survivors. There are several benefits associated with using yeast for genetic selection. In the first place, yeast is likely to be the simplest, fast-growing organism whose viability depends on
functional actin. Secondly, recombinant DNA technology and transformation systems in yeast are very well established. Finally, since actin-ADP-ribosylation by Vip2 is most likely responsible for toxicity in transgenic corn, it is reasonable to assume that, as an eukaryote, yeast can mimic this situation to a certain extent and provide informative experimental data from engineering efforts in a much shorter time than afforded by transgenic plants.

In order to test yeast cells for functional selection of Vip2 variants, both wild-type and the non-functional active-site mutant (E428G) genes were cloned into two yeast expression systems: high-copy number pYES2 and low-copy number p416GALS expression vectors. Both constructs were transformed into a laboratory strain of *S. cerevisiae* and selected under conditions supporting leaky expression from the GAL promoter (plates utilizing raffinose as a carbon source). Although E428G mutant gene in both expression systems produced plenty of yeast transformants, there were no visible colonies after transformation of wild-type Vip2 gene into yeast (Fig. 2). The E428G mutant *vip2* gene has thus served as a positive control to establish this system as useful for functional selection of Vip2 variants. Thus, this simple genetic system can likely be adopted for rapid screening of functional significance of amino acid residues in any actin-ADP-ribosyltransferase and for identification of critical residues. We considered that an actin-ADP-ribosyltransferase and for identification of critical amino acids is huge (20⁰²¹ variants). This complexity is vastly reduced by yeast transformation efficiency, which is the limiting factor in the whole screening/selection process. Thus, in reality, only a very small fraction of the generated

**Random elongation mutagenesis and selection of core propeptide sequence**

In order to attach a propeptide library to the C-terminal end of Vip2-ADP-ribosyltransferase, a recognition site for AatII restriction endonuclease was created at the end of the *vip2* gene. This modification changes the last amino acid of Vip2 into serine (N462S), without compromising toxicity in yeast. Therefore, in this report we refer to this Vip2 mutant as ‘wt’ (equivalent to wild-type).

A library encoding for random peptides (21-mers) was attached, via the engineered AatII site, to the 3′ end of *vip2* gene in the yeast low-copy number plasmid pMJ7 (p416GALS backbone). After yeast transformation, several colonies were selected under condition of ‘leaky’ expression from GAL promoter on plates supplemented with raffinose. Since pMJ7 plasmid carrying *vip2* gene does not produce transformants on raffinose plates, any surviving colonies are expected to harbour defective Vip2 toxin. In order to confirm the protective role of selected propeptides in Vip2 silencing, propeptides were recloned into pMJ7 plasmid backbone and re-tested in yeast transformation. Peptide from construct 4–12, VGWVPSRGEVFSLWVHGGWAR, was able to attenuate Vip2 activity to the extent that it allowed yeast colonies to emerge after transformation (although colonies exhibited signs of severe pathology, such as very slow growth). Furthermore, transformation efficiency with construct 4–4–12 was very low. Other peptides selected in the primary experiment did not pass our recloning test and appeared to be false positives. That is, colonies which originally survived after selection were most likely due to a novel mutation, deletion or rearrangement within *vip2* gene, rather than direct protection by the C-terminally attached peptides.

How probable is it to find a propeptide sequence with an inhibitory potential toward Vip2? The theoretical number of total sequences created by simultaneous randomization of 21 amino acids is huge (20⁰²¹ variants). This complexity is vastly reduced by yeast transformation efficiency, which is the limiting factor in the whole screening/selection process. Thus, in reality, only a very small fraction of the generated
sequence space can be screened by yeast transformation (library complexity is much higher than its ‘screenability’ in yeast). We conservatively estimate that sequences with some inhibitory potential to Vip2 can be selected (identified) from a population of 20,000–50,000 variants. Survivors attributable to the desired peptide extension (true positives) are rare – as they are diluted in the background of other survivors (false positives). For example, in our experiments only 8–15% of all selected survivors carried an ‘extension’. Deletions, rearrangements, mutations, etc. within the Vip2 gene are most likely responsible for this persistent background of survivors. In our selection strategy, we pursued the first ‘champion’ or confirmed survivor (as it remained after recloning and transformation) variant 4-4-12. Based on the fraction of initial survivors that we recloned, we estimate that this variant was selected after screening a population of ~4000–10,000 of sequences.

Semi-rational evolution of propeptide sequence

The spectrum of amino acids in the selected 4-4-12 propeptide (Fig. 3) does not correspond to the probability with which individual amino acids would be expected to appear in a random event. For example, in NN(G/C) randomization, the position of interest is changed to a complete set of 20 amino acids. Due to the disparity between residues such as Met and Trp, which have a single codon, and residues such as Leu, Arg, and Ser which have three codons, the probability with which individual amino acids appear in a completely unbiased library is different (Leu, Arg and Ser three times more frequently than Trp and Met). The presence of three tryptophans in propeptides of surviving clones suggests their putative importance for propeptide function. Conversely, some multiple codon residues (Arg, Leu, Ser, Ala, Pro) have been selected with lower frequency, which may reflect their lower information content (higher replaceability, lower importance) in the selected peptide. These analyses allowed us to postulate critical residues of the propeptide before attempting to improve its Vip2 protecting function by further mutagenesis.

In order to further improve properties of the selected propeptide, the 4-4-12 variant was subjected to an additional round of mutagenesis, in which blocks of several, presumably less important amino acids (PSR, SL, AR) were randomized simultaneously and subcloned into pMJ7. Due to the large number of yeast survivors after the second round of mutagenesis and a low proportion (~8%) of true positives (survivors containing a propeptide extension confirmed by recloning), all colonies were pooled and a mixed plasmid population was isolated from the pool. The plasmid pool was used as a template for PCR amplification of propeptide sequences, which were subsequently recloned into pMJ7 and propagated in E. coli. We anticipated that propeptide(s) with highest Vip2 inhibitory potential might be overrepresented in a recloned population, since they likely were overrepresented in an original population of yeast survivors in the form of largest (best-growing) colonies and/or multiple colonies. Plasmids isolated from individual E. coli colonies were re-tested in yeast for their ability to support the growth. As the parental, 4-4-12 proenzyme variant is able to form small colonies in yeast, we used a colony-size visual screen to identify propeptides with improved function. Two healthy colonies with noticeably improved growth have been identified among the first re-tested, recloned variants.

Interestingly, DNA sequencing of propeptide coding regions from both healthy survivors revealed the presence of: (i) a single nucleotide transversion (A to T) responsible for a Glu to Val substitution of the ninth amino acid in the propeptide region and (ii) a frameshift due to one nucleotide insertion after the eleventh amino acid (Phe) of the propeptide region thus extending the length of selected propeptides from the intended 21 to 49 amino acids. Part of these propeptides has thus been ‘acquired’ from translated DNA sequence located downstream of the vip2 gene itself. In codon randomization experiments, standard reaction conditions using Taq polymerase were used. Taq polymerase is known to be an error-prone enzyme that produces single-base substitution errors at a rate of 1 for each 9000 nucleotides polymerized and frameshift errors at a frequency of 1/41 000 (Tindall and Kunkel, 1988). The two selected propeptides have almost identical sequence, with only one conservative amino acid disparity (Thr versus Ala; Fig. 3) at position #39 of the polypeptide extension. Vip2 protein with the selected propeptide attached to the C-terminal end was designated proVip2. Removal of engineered propeptide-coding sequences from proVip2 gene restored lethality of Vip2-ADP-ribosyltransferase in yeast, confirming an indispensable function of these sequences for silencing the enzymatic activity of Vip2 in yeast. Functionality of propeptide sequence to compromise Vip2 toxicity was further confirmed by subcloning of propeptide sequences from low-copy number Vip2 plasmid backbone (pMJ7) into high-copy number Vip2 plasmid backbone (pMJ5) and the ability of yeast to tolerate an even higher dose of Vip2 in cells. These in vivo experiments clearly demonstrate that information necessary for yeast survival after transformation with Vip2 constructs resides on a propeptide sequence. After selection and confirmation of the colony (ultimately identified as ‘proVip2’) no further screening efforts were pursued.

![Propeptide sequences selected after mutagenesis. Core propeptide sequence (4-4-12) selected after randomizing of 21 amino acids residues and proVip2 sequence selected after second round of mutagenesis. A single nucleotide mutation (A to T) is responsible for substitution of the ninth amino acid (E to V) in the propeptide region. One nucleotide insertion acquired in a process of PCR amplification is responsible for a frameshift and extension of polypeptide chain from 21 to 49 amino acids. Point of frameshift (*) occurred after amino acid #11 (F) of the polypeptide chain extension.](https://academic.oup.com/peds/article-abstract/21/10/631/1494029/635?redirectedfrom=fulltext&pdfAccessKey=372AD6331184D47D3E9584544C093B16)
In vitro activity of Vip2 zymogens

From our in vivo selection in yeast it seemed obvious that the lethal effect of Vip2-ADP-ribosyltransferase in its zymogenic forms (proVip2) was compromised by C-terminally attached propeptides. To validate this further, however, it was necessary to express the proteins and demonstrate that Vip2 zymogen actually has a lower actin-ADP-ribosylating activity than the wild-type form of the protein. Both proteins, Vip2 and engineered proVip2, were expressed in E. coli BL21(DE3) cells from the pET29a system, and the ADP-ribosylation reaction performed in vitro with a non-muscle actin. Experiments to document the time course of ADP-ribosylation with Vip2 and proVip2 confirmed that the zymogenic proVip2-ADP ribosylates actin to a lesser extent than the wild-type protein (Fig. 4). Based on signal intensity, it was estimated from several independent experiments, that proVip2 exhibits <10% of actin-ADP-ribosylation activity of its parental, ‘wt’ form. These in vitro experiments confirmed that the interpretation of our genetic selection strategy in yeast in terms of decreased ADP-ribosylation activity of Vip2 variants was correct. Critically, even though proVip2 possesses <10% enzymatic activity of its native form, it retains potent toxicity to WCRW larvae. Incorporation of the mixture of Vip1 helper protein and proVip2 culture extracts into artificial diet caused 100% mortality of rootworm larvae in 72 h.

Detection of residual activity of Vip2 zymogen in planta

Our propeptide and construct design permitted growth of transgenic Vip2 plants to maturity for the first time. Before the engineering efforts presented in this work, it was not possible to grow transgenic plants with a Vip2 gene from maize transformation experiments. First generation of proVip2 transgenic corn did not show any symptoms of plant pathology under greenhouse conditions and was phenotypically unrecognizable from the control, untransformed plants. In order to confirm the presence of proVip2 in transgenic corn, we performed the enzymatic ADP-ribosyltransferase assay with plant root extracts. In this sensitive labeling assay, we were able to detect ADP-ribosylation activity in root extracts from corn plants transformed with proVip2 (Fig. 5). Non-transgenic maize had no detectable actin-ADP-ribosylation activity. Also, presence of the Vip2 antigen contained within maize proVip2 plants was detectable by the anti-Vip2 antibody (data not shown). We have estimated the amount of proVip2 present in transgenic material at 5–40 ng per mg of total soluble protein.

Engineered Vip2 zymogen can be processed and activated by rootworm larvae

As the primary goal of this study was to design a genetic system for selection of inactive Vip2 mutants and test this system for selection of ‘zymogenized’ (polypeptide chain extended and malfunctional) Vip2 variants, we should consider attributes of an ‘ideal’ zymogen. First, the ADP-ribosylating activity of ‘zymogenized’ Vip2 must be low enough to be tolerated by the plant host without symptoms of aberrant phenotype. Survival in planta supports the first criterion. Secondly, the Vip2 zymogen should either possess enough residual enzymatic activity to be toxic to the plant pest, or—in an ideal scenario—have a potential to be converted into an enzymatically active form by rootworm digestive machinery.

So in order to explore the digestive fate of proVip2 and assess this aspect of its zymogen behavior, we designed a rootworm feeding assay. As rootworm larvae possess a broad assortment of digestive enzymes (Bown et al., 2004), we further explored if engineered proVip2 could be processed and possibly activated to the wild-type form in the rootworm digestive system.

To facilitate visualization of protein after digestion, high doses of Vip2 proteins were incorporated into insect diet, achieved by using concentrated extracts from 10 ml of E. coli cell culture.

For Vip2 protein detection in whole body homogenates, rootworm larvae were fed on artificial diet comprising Vip2 proteins for 30 or 90 min. After feeding, extracts from homogenized WCRW larvae were separated by SDS–PAGE and analyzed by Vip2 antibody detection on the western blot (Fig. 6). Importantly, we found that engineered Vip2 proenzymes, with or without an S-tag at the N-terminus (proVip2 and S-tag-proVip2), can be processed to a stable form of approximately the same size as Vip2 by WCRW larvae. In

Fig. 5. Demonstration of ADP-ribosylation activity in root extract from transgenic proVip2 plant. Extraction of root proteins and ADP-ribosylation reaction were performed as described in ‘Materials and methods’. Aliquots of enzymatic reaction were taken out at different time points (1, 3, 5, 15, 60 min) and subjected to SDS–PAGE. After blotting onto PVDF membrane, ADP-ribosylated actin was visualized by autoradiography.

Fig. 6. Digestive fate of Vip2 proteins in WCRW. Vip2 variants detected in WCRW whole body homogenates after feeding for 30 and 90 min. Lane – (1) S-tag-proVip2 (30 min), (2) S-tag-proVip2 (90 min), (3) proVip2 (30 min), (4) proVip2 (90 min), (5) S-tag-Vip2 (30 min), (6) S-tag-Vip2 (90 min), (7) Vip2 (30 min), (8) Vip2 (90 min). Closed arrows denote putative activated form of proVip2 proteins co-migrating with Vip2 (open arrow).
expression of Vip2 protein in plants is lethal and thus cannot be used for transgenic purposes. On the other hand, a created zymogen would need to be activated by the digestive machinery of the target pest in order to exert its lethal function. Undoubtedly, this is a very complex problem and the main goal of this report was to design feasible tools and a model strategy on how to deal with this problem. At first, we devised a simple in vivo system for selection of defective Vip2 variants in yeast. This strategy can be easily adopted for rapid screening to determine potential functional significance of amino acid residues in any actin-ADP-ribosyltransferase and for identifying these critical residues. Using random elongation mutagenesis at the C-terminus of the protein and selection in yeast, we identified a Vip2 proenzyme with significantly reduced enzymatic activity and ability to survive in corn plants without causing developmental pathology under greenhouse conditions. Moreover, the engineered zymogen is still powerful enough to cause rootworm mortality, most likely due to activation in the rootworm digestive system to the wild-type enzymatic form.

In this manuscript, we created zymogen-like form of a toxic protein by extension of its polypeptide chain. What role does the engineered propeptide region play in executing its protecting function?

At the time of designing experiments presented in this work, we presumed that proper extension of the polypeptide chain of Vip2-ADP-ribosyltransferase could interfere with its enzymatic function by at least four potential mechanisms (see Results and discussion). Our work has not yet pinpointed the exact mechanism by which the propeptide region executes its protective effect. Likely, a small change to one or all of the four potential mechanisms (and possibly some other) is actually responsible for the observed effects. Although our design and selection approach supports use of the zymogen strategy, the complete solution to Vip2 expression challenges in planta would involve a further decrease (or ideally complete elimination) of ADP-ribosyltransferase activity.

Most zymogens have their propeptides localized at the N-terminus, which seems to be logical considering that synthesis of the propeptide region precedes that of the catalytic unit, thus preventing any undue activation of the zymogen (Lazure, 2002). Hence, for example, it has been reported that the C-terminal pro-sequence of the subtilisin-type serine protease from Thermus aquaticus, Aqualysin I, retards the proteolytic activation of the precursor (Lee et al., 1992). Other roles have been associated with C-terminal propeptides, including extracellular secretion (Pohlner et al., 1987; Kim et al., 1997) and involvement in protein folding (Ohnishi et al., 1994).

During the course of this work, Plainkum et al. (2003) reported the creation of a zymogen from ribonuclease A by circular permutation and introduction of a highly specific protease site into a short peptide linking N- and C-termini. Similar strategy has been used in another report from the same laboratory (Johnson et al., 2006). In the case of Vip2-ADP-ribosyltransferase, N- and C-termini are far apart which could make it difficult to circularly permutate its polypeptide chain with a short peptide linker. Moreover, engineered Vip2 zymogen “in action” will be exposed to the whole set of proteolytic enzymes in rootworm digestive system. Accordingly, a Vip2 zymogen has to be at least marginally

**Conclusion**

In nature, potentially toxic enzymes are often synthesized in a non-functional, inactive state. The generation of nascent enzyme precursors (proenzymes,zymogens) is well known in the family of proteolytic enzymes. When inactive proenzymes reach the right place at the right time, they are usually subjected to activation by other proteases or in an autocatalytic manner. In this report, we attempted to create a zymogen of Vip2-ADP-ribosyltransferase to explore avenues for reducing phytotoxicity when expressed in planta. As Vip2 ribosylates one of the most conserved proteins in nature, it is reasonable to assume that this toxin would likely be toxic to any cells requiring actin for their viability. In its native form, the case of the N-terminally tagged Vip2 protein (S-tag-Vip2) processing involved removal of the S-tag [as determined by detection with S-protein antibody, where the processed bands lacked the signal (data not shown)]. These data support the interpretation that WCRW larvae can activate the proVip2 molecule upon ingestion.

For Vip2 protein detection in frass, rootworm larvae were fed artificial diet incorporated with Vip2 proteins for 3 days, then frass was collected and analyzed for the presence of enzymatic activity and to reveal any proteolytic processing which took place. Since the other protein component of binary toxin (Vip1) was not incorporated into the diet, feeding with Vip2 protein alone for longer period of time (3 days) did not cause feeding inhibition or larval mortality. Analysis of proVip2 processing and enzymatic activity in frass from insect larvae again clearly demonstrated that enzyme precursors could be proteolytically processed to a stable, activated form of the protein. A substantially smaller amount of processed proVip2 protein recovered from rootworm frass had greater enzymatic activity than a much larger amount of undigested, control proVip2 protein (Fig. 7). These data therefore suggest that complete or partial removal of the engineered C-terminal peptide present in proVip2 by WCRW proteolytic activity has effectively ‘unmasked’ the enzymatic activity needed to confer toxicity.

**Fig. 7.** Enzyme assay (A) and western blot (B) of engineered enzyme precursors (lanes 2 and 4) and their processed forms collected from WCRW larvae frass (lanes 3 and 5) after 3-day feeding. Lane – (1) MW marker, (2) proVip2, (3) proVip2 collected from frass, (4) S-tag-proVip2, (5) S-tag-proVip2 collected from frass, (6) Vip2, (7) Vip2 collected from frass.

Enzyme assay (A) and western blot (B) of engineered enzyme precursors (lanes 2 and 4) and their processed forms collected from WCRW larvae frass (lanes 3 and 5) after 3-day feeding. Lane – (1) MW marker, (2) proVip2, (3) proVip2 collected from frass, (4) S-tag-proVip2, (5) S-tag-proVip2 collected from frass, (6) Vip2, (7) Vip2 collected from frass.

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stable and possibly activatable in this harsh environment in order to impart toxicity. Due to the complexity of the problem, our strategy relied on a ‘less rational’ engineering approach for zymogen design, involving random extension of a polypeptide chain and selection in yeast. The selected proenzyme proved to be benign in transgenic plants under greenhouse conditions and can be processed and activated in vivo by pest digestive proteases. This report thus represents another example of applying the protein engineering approach for zymogen creation that may outline a more general strategy for solving certain challenges of using toxic proteins in biotechnology research and applications.

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