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# Processing of a plant peptide hormone precursor facilitated by post-translational tyrosine sulfation

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**This file includes:**

Main Text  
Figures 1 and 2

## Abstract

Most peptide hormones and growth factors are matured from larger inactive precursor proteins by proteolytic processing and further post-translational modification. Whether or how post-translational modifications contribute to peptide bioactivity is still largely unknown. We address this question here for TWS1 (Twisted Seed 1), a peptide regulator of embryonic cuticle formation in *Arabidopsis thaliana*. Using synthetic peptides encompassing the N- and C-terminal processing sites and the recombinant TWS1 precursor as substrates, we show that the precursor is cleaved by the subtilase SBT1.8 at both the N- and the C-terminus of TWS1. Recognition and correct processing at the N-terminal site depended on sulfation of an adjacent tyrosine residue. Arginine 302 of SBT1.8 was found to be required for sulfo-tyrosine binding and for accurate processing of the TWS1 precursor. The data reveal a critical role for post-translational modification, here tyrosine sulfation of a plant peptide hormone precursor, in mediating processing specificity and peptide maturation.

## Main Text

### Introduction

In plants and animals alike, peptide hormones and growth factors are derived from larger precursor proteins by post-translational processing and modification. Peptides with different, even antagonistic activities may be produced from the same precursor, highlighting the relevance of post-translational maturation events (1-4). Secretion, stability, and metabolic clearance of peptide hormones may also be regulated post-translationally (4). The activity of human cholecystokinin and of phytosulfokine (PSK) and Casparian strip Integrity Factors (CIFs) in plants, for example, depends on post-translational tyrosine sulfation to facilitate receptor binding and activation (5-7). Here, we show an additional role for tyrosine sulfation as a critical step in peptide hormone biogenesis. We found that sulfo-tyrosine determines the specificity of processing of the Twisted Seed 1 (TWS1) precursor. Only in its tyrosine-sulfated form is the precursor cleavage site recognized and accurately processed by subtilase (SBT) 1.8.

TWS1 belongs to the CIF family of sulfated peptides (Fig. 1A) that control Casparian strip and embryonic cuticle formation. The Casparian strip is an apoplastic diffusion barrier regulating water and ion homeostasis in roots. It forms a continuous impermeable band around radial cell walls of the endodermis, preventing apoplastic movement of water and nutrients from the cortex into the root stele. Its integrity is controlled by CIF1 and CIF2 that are produced and secreted from the stele (8, 9). The mature peptides diffuse extracellularly into adjacent tissues. Perception in the endodermis by the polarly localized GASSHO1/SCHENGEN3 (GSO1/SGN3)-SGN1 receptor complex triggers spatially controlled lignification for continued Casparian strip formation. Upon completion, the Casparian strip isolates the receptor complex from the source of the signal, and signaling is thus terminated (8, 9). Integrity of the embryonic cuticle is monitored by a similar TWS1-dependent signaling pathway with, however, an interesting twist. Other than CIF1 and CIF2, TWS1 bears an inhibitory C-terminal extension (Fig. 1A). It is secreted from the embryo in this inactive form and passes through the nascent cuticle into the seed endosperm, where the C-terminal extension is cleaved off by the subtilase ALE1 (10). Fully matured TWS1 is then perceived by the GSO1 and GSO2 receptors at the embryo surface, and the activated receptors signal for continued cuticle production. Only when all the gaps are filled, the now impermeable cuticle separates the TWS1 precursor peptide from its activating protease, and the signaling pathway is shut off (10).

### Results and Discussion

In contrast to C-terminal processing and the critical role of ALE1 in the activation of TWS1, the proteases responsible for N-terminal maturation of CIF family peptides are still elusive. The N-terminus of CIFs is marked by a conserved DY motif (Fig. 1A) and sulfation of tyrosine in P2' (the

second position downstream of the cleavage site) by tyrosyl protein sulfotransferase is required for bioactivity (10). Aiming to identify the N-terminal processing enzyme for TWS1, we searched publicly available databases for proteases that are co-expressed with ALE1 in developing Arabidopsis seeds (Dataset S1), and identified SBT1.8 as a possible candidate.

Promoter:reporter gene analysis confirmed expression of SBT1.8 in the endosperm, in addition to weaker expression in the suspensor and provascular tissues of the embryo (Fig. 1B). Synthetic peptides encompassing the N-terminal (E~DY; sulfo-tyrosine underlined) and C-terminal (H~G) processing sites of TWS1 were tested as substrates for SBT1.8. The E~DY-bond was efficiently cleaved by SBT1.8 resulting in the correctly processed N-terminal TWS1 peptide as the main cleavage product (Fig. 1C, upper panel). The second peptide was trimmed by SBT1.8 from the C-terminus up to the H~G-bond at the C-terminal processing site (Fig. 1C, lower panel). The data suggest that SBT1.8 cleaves the TWS1 precursor at both the N- and the C-terminal processing sites to produce the mature TWS1 peptide.

In order to confirm this result, we tested the full-length TWS1 precursor produced as a GST fusion in *E. coli* as substrate of SBT1.8. A single cleavage product was observed for the GST-TWS1xSBT1.8 digest that was identical in size to that produced by ALE1 (Fig. 1D). Cleavage at the C-terminal H~G-bond was confirmed by mass spectrometry (Fig. 1E). The data suggest that SBT1.8 acts redundantly with ALE1 in C-terminal maturation of TWS1. Surprisingly, however, processing at the N-terminal site, as seen for the synthetic peptide (Fig. 1C), was not observed in this experiment.

Puzzled by this discrepancy, we pondered whether sulfo-tyrosine that was included in the peptide substrate but lacking from the recombinant precursor might be important for cleavage by SBT1.8. Hence, we produced the tyrosine-sulfated precursor using an expanded genetic code in *E. coli*. Briefly, the relevant tyrosine codon within the GST-TWS1 expression construct was replaced by an amber stop codon. The construct was then co-expressed in *E. coli* with a suppressor tRNA recognizing the amber stop, and a matching aminoacyl-tRNA synthetase specific for sulfo-tyrosine, which was added to the growth medium (11). Consequently, sulfo-tyrosine is co-translationally incorporated at the genetically encoded position, resulting in the production of full-length sulfated GST-sTWS1, in addition to a truncated protein terminated at the amber stop (Fig. 2A). Upon digestion with SBT1.8, the C-terminal cleavage product was observed again (Fig. 2A, blue arrow), in addition to a novel band corresponding in size to the N-terminal cleavage product (Fig. 2A, red arrow). The data suggest that sulfation of P2' tyrosine enables recognition of the N-terminal site and cleavage by SBT1.8. This conclusion was confirmed by use of sulfated and non-sulfated synthetic peptides to directly compare the effect of tyrosine sulfation on cleavage efficiency. SBT1.8 produced the correctly processed peptide from the sulfated substrate (sTWS1; Fig. 2B), with only a minor contamination of a second peptide, resulting from mis-cleavage after the sulfo-tyrosine residue. In contrast, for the non-sulfated substrate (TWS1), the mis-cleaved peptide was the main reaction product. This mis-cleavage was in fact also observed for the non-sulfated GST-TWS1 precursor protein, albeit only at longer incubation times, or higher concentration of the protease (Fig. 2C). The data suggest that tyrosine sulfation prevents mis-cleavage by increasing the specificity of processing, directing SBT1.8 to the correct N-terminal processing site.

We then used structural modelling to find out how the sulfate may contribute to substrate binding and cleavage site recognition. Figure 2D shows the sulfated substrate peptide modelled into the active site of SBT1.8. The model suggests a critical role for R302 in substrate binding as it interacts with the negatively charged sulfonate of the P2' sulfo-tyrosine residue (Fig. 2D, Movie S1, Dataset S2). Site-directed mutagenesis confirmed the relevance of R302 for substrate recognition. N-terminal cleavage of the GST-sTWS1 precursor was no longer observed when R302 was substituted with lysine, methionine, serine, glutamate, valine, or isoleucine (Fig. 2E). Importantly, activity of SBT1.8 *per se* was not affected by these mutations, as C-terminal cleavage was not impaired (Fig. 2E). The data indicate that R302 is required for cleavage at the correct N-terminal processing site.

However, R302 is not solely responsible for cleavage site recognition, as SBT1.1 and SBT5.4, which also feature arginine in the corresponding position, fail to cleave the GST-sTWS1

precursor at the N-terminal processing site (Fig. 2F). Additional specificity may be provided by S333. Modeling suggests that the side chains of R302 and S333 interact with Asn in P3' of the TWS1 substrate (Fig. 2D, Movie S1). S333 is not conserved, neither in SBT1.1 nor in SBT5.4, thus explaining why these proteases fail to cleave TWS1 at the N-terminal site. While SBT1.1 and SBT5.4 are clearly not involved, other proteases are present (Dataset S1) and may contribute to TWS1 formation in the endosperm. Interestingly, the P3' Asn residue is uniquely found in TWS1, and replaced by Gly in other CIF peptides (Fig. 1A). This observation suggests that SBT1.8 interacts specifically with TWS1 and may not be involved in the processing of the CIF peptides regulating Casparian strip formation.

We report here an Arabidopsis protease (SBT1.8) that specifically recognizes the post-translational modification (tyrosine sulfation) of its peptide precursor substrate (proTWS1). Tyrosine sulfation prevents mis-cleavage of proTWS1, suggesting that the sulfo-tyrosine-dependent interaction with SBT1.8 evolved to increase the specificity of precursor processing for the biogenesis the TWS1 peptide. Post-translational modification of peptide hormones, particularly tyrosine sulfation of TWS1 and of other sulfo-peptides in plants and animals alike, was previously shown to be required for high-affinity binding to cognate receptors, and hence, for peptide activity (5-7, 10). We show here that post-translational modification, as a prerequisite for accurate precursor processing, may also contribute to bioactivity by controlling peptide hormone biogenesis.

## Materials and Methods

Light microscopy for reporter gene analysis was performed as described (12). Expression and purification of recombinant proteases and substrate proteins, and mass spectrometric analysis of cleavage products were performed as in ref. (10). Structural modeling followed the strategy outlined in ref. (13) with tomato SBT3 as template structure (14). Procedures are detailed in SI Appendix, Extended Methods.

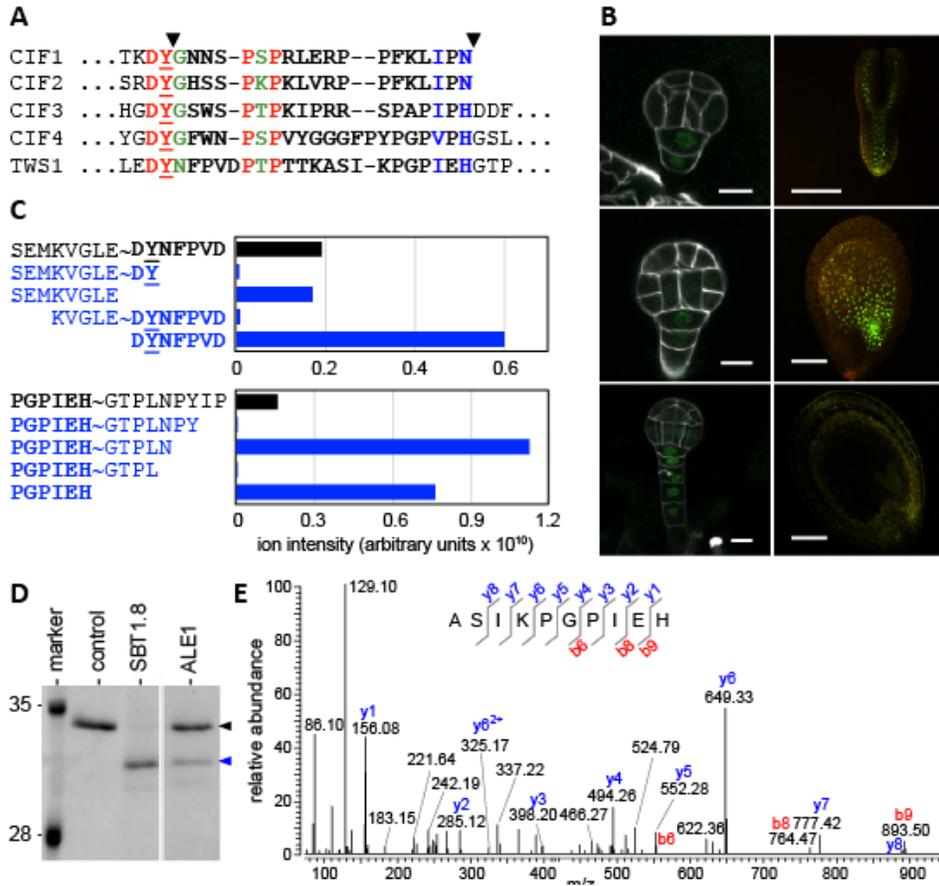
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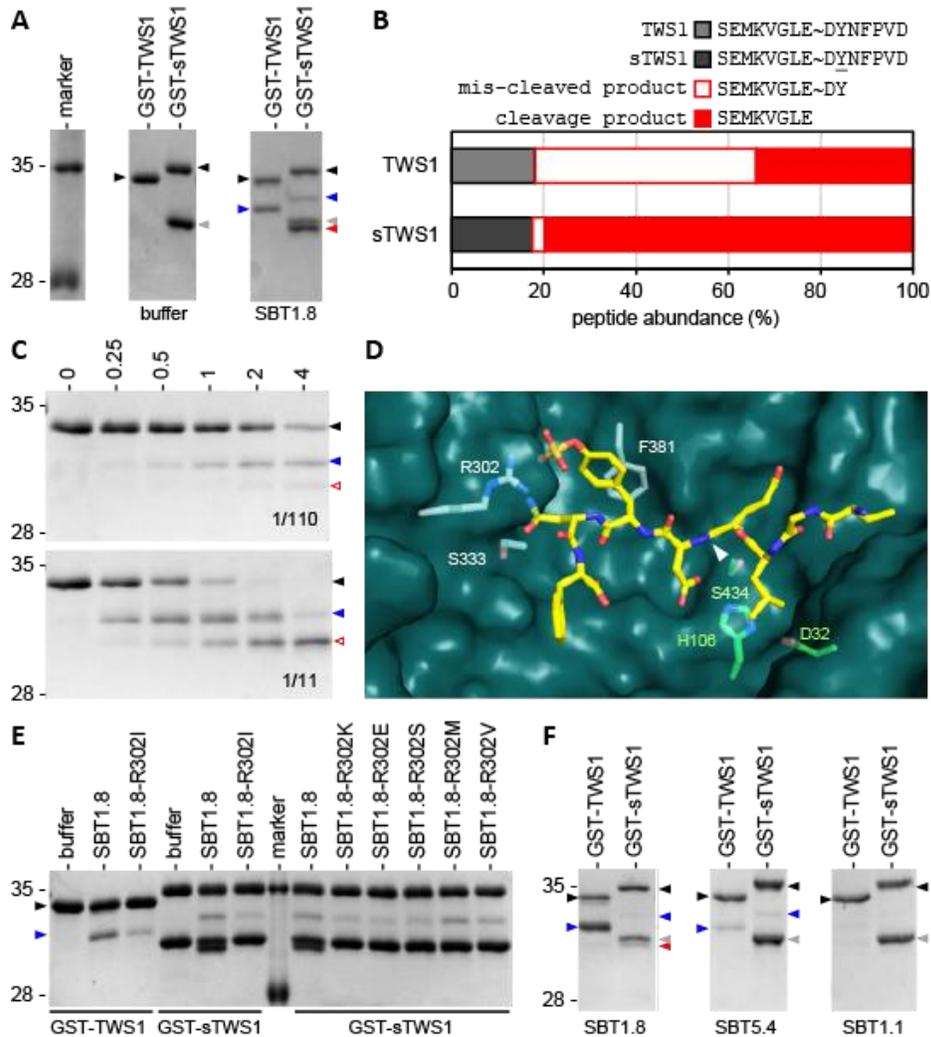
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**Figure 1.** Processing of the TWS1 precursor by endosperm proteases ALE1 and SBT1.8. (A) Alignment of Arabidopsis CIF family peptide precursors with N- and C-terminal maturation sites indicated by arrowheads; Y = sulfo-tyrosine; mature peptides in bold. (B) Promotor:reporter gene analysis of *SBT1.8* expression (green) during Arabidopsis embryo and seed development; bar = 10  $\mu$ m (left) or 100  $\mu$ m (right column). (C) Mass spectrometry (MS) cleavage assay with synthetic peptide substrates (sequence of substrate peptides black, cleavage products blue) including N-terminal (E~DY; top) and C-terminal (H~G; bottom) processing sites. Bar graphs show the abundance (peak ion intensity) of peptide cleavage products (blue) and of residual substrate peptides (black). (D) SDS-PAGE analysis of cleavage products (blue arrow) generated from recombinant GST-TWS1 (black arrow) by SBT1.8 and ALE1. (E) MS/MS identification of the cleavage product produced by SBT1.8 in (D). Identity and sequence of the C-terminal peptide was confirmed by the almost complete y-ion series (blue), and additional ions from the b series (red).



**Figure 2.** Tyrosine sulfation facilitates N-terminal cleavage by SBT1.8. (A) Cleavage of GST-TWS1 and sulfated GST-sTWS1 by SBT1.8. In the buffer control, full-length and truncated forms of the GST-sTWS1 precursor are marked by black and gray arrowheads, respectively. Blue and red arrowheads mark the C-terminal and N-terminal cleavage products in the SBT1.8 digest. (B) MS assay comparing relative abundance (%) of cleavage products (30 min digest) for the sulfated (sTWS1) and unmodified (TWS1) N-terminal precursor peptides. Residual substrate peptides in gray, correctly processed and mis-cleaved peptides as full and open red bars, respectively. (C) Time course of GST-TWS1 cleavage at a molar protease:substrate ratio of 1:110 (top) and 1:11 (bottom). (D) Substrate peptide (yellow stick) including the N-terminal processing site (arrowhead) modeled into the active site of SBT1.8. Residues of the catalytic triad (D32, H106, S434; numbering of residues refers to the mature protease lacking the prodomain) are shown in green. P2' sulfo-tyrosine interacts by  $\pi$ -stacking with the aromatic ring of F381 in the hydrophobic S2' pocket, and via the sulfate moiety with R302 of SBT1.8. The side chain of P3' Asn hydrogen-bonds with R302 and S333. (E, F) SDS-PAGE analysis of cleavage products generated from GST-TWS1 and GST-sTWS1 by SBT1.8 and site-directed R302 mutants (E), and by SBT5.4 and SBT1.1 (F). Protein bands corresponding to C- and N-terminal cleavage products are indicated by blue and red arrowheads, respectively.