Proteasome activity imaging and profiling characterizes bacterial effector Syringolin A

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Syringolin A (SylA) is a non-ribosomal cyclic peptide produced by the bacterial pathogen Pseudomonas syringae pv. syringae that can inhibit the eukaryotic proteasome. The proteasome is a multi-subunit proteolytic complex that resides in the nucleus and cytoplasm and contains three subunits with different catalytic activities: β1, β2 and β5. Here, we studied how SylA targets the plant proteasome in living cells using activity-based profiling and imaging. We further developed this technology by introducing new, more selective probes and establishing procedures of non-invasive imaging in living plant cells. These studies showed that SylA preferentially targets β2 and β5 of the plant proteasome in vitro and in vivo. Structure-activity analysis revealed that dipeptide tail of SylA contributes to β2 specificity and identified a nonreactive SylA derivative that proved essential for imaging experiments. Interestingly, subcellular imaging with probes based on epoxomicin and SylA showed that SylA accumulates in the nucleus of the plant cell and suggests that SylA targets the nuclear proteasome. Furthermore, subcellular fractionation studies showed that SylA labels nuclear and cytoplasmic proteasomes. The selectivity of SylA for the catalytic subunits and subcellular compartments is discussed and the subunit selectivity is explained by crystallographic data.
INTRODUCTION

The interaction between Arabidopsis thaliana and the bacterial pathogen Pseudomonas syringae is an important model system to study plant-pathogen interactions. Besides Arabidopsis, strains of this bacterial pathogen cause various diseases on a wide range of plant species, including fruit trees, tomato and other crop plants (Hirano and Upper, 2000). P. syringae manipulates its host by injecting effector proteins through the type-III secretion system into the host cell (Boller & He, 2009; Buttner & He, 2009; Cunnac et al., 2009; Goehre & Robatzek, 2008; Lewis et al., 2009). Many of these effectors suppress basal defense responses (Guo et al., 2009; Cunnac et al., 2009).

Besides type-III effectors, P. syringae strains also produce different small molecule effectors to manipulate the host. Coronatine from P. syringae pv. tomato DC3000, for example, induces the jasmonate signaling cascade, provoking e.g. the opening of stomata to overcome pre-invasive immunity (Melloto et al., 2006). Other examples are tabtoxin from P. syringae pv. tabaci, which inhibits glutamine synthetase; and phaseolotoxin from P. syringae pv. phaseolicola, which causes arginine deficiency by inhibiting ornithine carbamoyl transferase (Bender et al., 1999). It is evident from these studies that small molecule effectors are equally important for bacterial diseases as type-III effectors. Yet, studies on small molecule effectors and their targets are limited.

Here, we study the action of syringolin A (SylA), a small molecule effector produced by Pseudomonas syringae pv. syringae (Psy). SylA is a non-ribosomal cyclic peptide that contributes to the development of disease symptoms (Groll et al., 2008). SylA is a 493 Da molecule that consists of a 12-membered macrocycle and a dipeptide tail (Wäspi et al., 1999). The ring contains an α,β-unsaturated amide and a second double bond. This second unsaturated bond is absent in SylB, a minor, additional metabolite produced by Psy (Wäspi et al., 1999). The dipeptide tail contains two L-valines, linked through an ureido bond. SylA is produced by Psy by non-ribosomal peptide and polyketide synthetases encoded by the sylC and sylD biosynthesis genes and presumably secreted from bacteria by the product of sylE, which encodes a transporter-like protein (Amrein et al., 2004; Ramel et al., 2009; Imker et al., 2009).

SylA inhibits the eukaryotic 26S proteasome (Groll et al., 2008). The 26S proteasome is a large, multicomponent protease residing in the cytosol and nucleus and consists of a 20S core protease and a 19S regulatory particle. The 19S regulatory particle accepts ubiquitinated substrates, unfolds them and feeds them into the core protease (Kurepa and Smalle, 2008). The
20S core protease consists of four rings of seven subunits each that make a hollow cylinder. The inner two rings consist of β subunits (Groll et al., 1997). The proteolytic activity is located in the inner cavity of the cylinder and resides in three β subunits of the inner two rings. Each of these subunits has slightly different catalytic activities: β1 cleaves after acidic residues (caspase-like activity); β2 after basic residues (trypsin-like activity); and β5 after hydrophobic residues (chymotrypsin-like activity) (Dick et al., 1998). Together, these subunits degrade proteins into 3-20 amino acid long peptides that are released into the cytosol or nucleus (Kurepa and Smalle, 2008).

Crystallographic data revealed that the α,β-unsaturated amide of SylA is attacked by the N-terminal threonine of the catalytic β subunits, resulting in an irreversible, covalent ether bond (Groll et al., 2008). Further studies showed that SylA has anti-apoptotic properties in mammalian cells and is therefore a promising novel anti-cancer drug, having different properties when compared to e.g. bortezomib, a proteasome inhibitor that is currently used in the clinic as an anti-cancer drug (Coleman et al., 2006; Clerc et al., 2009a).

That SylA inhibits the plant proteasome in vivo has been demonstrated by its ability to promote the accumulation of cyclin-GUS fusion proteins in root tips (Groll et al., 2008). Beyond this, little is known on how this small molecule effector interacts with its natural host target and if the proteasome is the only target in plants. Here, we study the action of SylA in plants using activity-based probes, which are reporter-tagged inhibitors that react with active site residues of enzymes in a mechanism-dependent manner. The irreversible covalent bond facilitates the display of labeled enzymes on protein gels, and/or the identification of labeled proteins by affinity capture and mass spectrometry (Cravatt et al., 2008). We recently introduced proteasome activity profiling in plants using MV151, a fluorescent vinyl sulfone probe that labels the β1, β2, and β5 subunits of the proteasome and several papain-like cysteine proteases (PLCPs) (Gu et al., 2010).

Activity-based Protein Profiling (ABPP) is a simple and robust approach that is now also used in plant science (Kolodziejek & Van der Hoorn 2010). In this study, we further developed the ABPP technology to facilitate detailed studies of the selectivity of SylA on its natural host target, the plant proteasome. To this end, we introduce novel, selective proteasome probes and established procedures for in vivo proteasome activity profiling and imaging. With these tools, and using SylA derivatives, we examined the subunit selectivity and subcellular targeting of SylA in living plant cells.
RESULTS
Comparison of three proteasome probes in vitro

In this study we used three different probes that target the proteasome (Figure 1A and Supplemental Figure S1). All three probes carry a fluorescent reporter tag but differ in their reactive group. MV151 contains a vinyl sulfone (VS), and has previously been used to investigate plant and animal proteasomes (Verdoes et al., 2006; Gu et al., 2010). MVB003 is based on the highly selective proteasome inhibitor epoxomicin and carries an epoxyketone reactive group (Meng et al., 1999). RhSylA is a fluorescent SylA derivative, previously used on animal extracts (Clerc et al., 2009a), carrying a reactive Michael system in the ring structure.

Each of these reactive groups binds covalently and irreversibly with the N-terminal active site threonine of the catalytic subunits of the proteasome, but through distinct molecular mechanisms (Groll et al., 2008; Huang and Chen, 2009). In addition to MVB003, we used two more epoxomicin-based probes, which only differ from MVB003 in the reporter tags. MVB070 contains bodipy carrying an azide minitag for click chemistry, and MVB072 contains bodipy carrying a biotin for affinity purification (Supplemental Figure S1). MVB003, MVB070 and MVB072 cause nearly identical labeling profiles (data not shown), but only MVB003 was used for in vivo experiments.

To determine in vitro labeling with the probes, extracts from Arabidopsis cell cultures were incubated with the probes for two hours. Proteins were separated on protein gels and analyzed for fluorescently labeled proteins by fluorescent scanning. MV151 labeling of Arabidopsis extracts causes three strong signals in the 25 kDa range, representing the catalytic subunits of the proteasome (Figure 1B, lane 2; Gu et al., 2010). MV151 labeling also causes weak signals at 30 and 40 kDa representing papain-like cysteine proteases (PLCPs, Gu et al., 2010). MVB003 and RhSylA labeling of extracts causes strong signals in the 25 kDa range, similar to those of MV151-labeled extracts (Figure 1B, lanes 3 and 4). MVB003-labeled proteins run slightly lower in the gel, probably because MVB003 has a smaller molecular weight compared to MV151 and RhSylA.

To investigate the potential subunit selectivity of the different probes, we performed time course labeling experiments in vitro. Labeling in extracts occurs within minutes for all three probes (Figure 1C). MV151 and MVB003 label β5 within one minute, followed quickly by β2, whereas β1 becomes labeled within 15 minutes (for MVB003) or 30 minutes (for MV151) (Figure 1C). In contrast, RhSylA labels β2 and β5 simultaneously within minutes, but β1 labeling takes one hour, which is longer when compared to MV151 and MVB003 labeling (Figure 1C). These data show that MV151 and MVB003 preferentially target β5, whereas RhSylA preferentially targets β5 and β2.
To further validate the subunit specificity of RhSylA, Arabidopsis leaf extracts were labeled for two hours with different concentrations of RhSylA. Both β2 and β5 reach maximum labeling at concentrations below 1 μM, whereas β1 labeling is not saturated even at 8 μM (Figure 1D), consistent with a slow labeling of β1. To determine if also SylA itself has low affinity for β1, Arabidopsis leaf extracts were preincubated with various SylA concentrations and then labeled with MVB072. This showed that labeling of β2 and β5 is prevented at low SylA concentrations (concentration for 50% inhibition (IC50) = 0.36 and 0.31 μM, respectively), whereas labeling of β1 requires high SylA concentrations (IC50 = 142.48 μM) (Figure 1E). Taken together, these experiments demonstrate that SylA and RhSylA preferentially target β2 and β5 subunits of the proteasome.

Comparison of three proteasome probes in vivo

To establish in vivo labeling in Arabidopsis cell cultures, the toxicity of the probes and inhibitors was first determined using Evans blue staining (Kaffarnik et al., 2008). Concentrations of 100 μM SylA or 20 μM epoxomicin caused significant cell death when incubated for 2.5 hours with cell culture (Figure 2A). Lower concentrations of inhibitors (50 μM SylA or 10 μM epoxomicin) or addition of probes (2 μM) did not affect cell viability and were used for in vivo inhibition assays (Figure 2A).

We next established procedures to prevent ex vivo labeling. In vitro proteasome labeling with the probes is so quick (Figure 1C) that a significant labeling occurs when probes are added during protein extraction (Figure 2B, lane 2). However, extraction in the presence of 1 or 2% SDS completely prevented ex vivo labeling (Figure 2B, lanes 3 and 4). Signals that appear after labeling in vivo, followed by extraction in 2% SDS are therefore only caused by in vivo labeling (Figure 2B, lane 5). For all subsequent in vivo labeling assays we used 2% SDS in the extraction procedure.

In vivo labeling of Arabidopsis cell cultures with MV151, MVB003 and RhSylA causes profiles that are different from the in vitro labeling profiles (Figure 2C). Most obvious are the relatively strong signals at 30 and 40 kDa in the MV151 profile (Figure 2C, lane 4). These data are consistent with the observation that PLCPs are more active in vivo when compared to in vitro (Kaschani et al., 2009). Second, the proteasome-derived signals are significantly weaker for all three probes when compared to in vitro labeling (Figure 2C), but this correlates with lower protein levels. However, despite differences in intensities, the profiles are not significantly changed when compared to in vitro labeling. MV151 and MVB003 still preferentially label β5 (Figure 2C, lanes 4 and 6), whereas RhSylA preferentially labels both β2 and β5 (Figure 2C, lane 8). Signals in the 30 and 40 kDa range or any other MW are absent
with MVB003 or RhSylA, indicating that these probes do not label PLCPs and are specific for the catalytic subunits of proteasome.

**In vivo** inhibition studies confirm the identities of labeled proteins

To verify the identity of the **in vivo** labeled signals, we preincubated cell cultures with various inhibitors and then labeled the cultures with the different probes. As inhibitors we used sublethal concentrations of SylA, E-64d, and epoxomicin. Preincubation with E-64d prevents MV151 labeling of the 30 and 40 kDa signals of MV151 (**Figure 2D**, lane 3), consistent with the fact that these signals are derived from PLCPs (Gu et al., 2010; Kaschani et al., 2009). Pretreatment with E64d has no influence on the labeling of MVB003 or RhSylA (**Figure 2D**, lanes 8 and 13). Pretreatment with epoxomicin or SylA selectively prevents labeling of all three 25 kDa signals in the MV151 profile (**Figure 2D**, lanes 4 and 5). In contrast, epoxomicin significantly suppresses overall MVB003 labeling (**Figure 2D**, lane 9) and prevents RhSylA labeling (**Figure 2D**, lane 14). Interestingly, SylA pretreatment suppresses MVB003-labeling of β5 and β2, but not β1 (**Figure 2D**, lane 10), and suppresses RhSylA labeling of all signals (**Figure 2D**, lane 15). In summary, these data confirm that the signals at 30 and 40 kDa are PLCPs and the signals at 25 kDa are from the proteasome. These data also support the previous notion that MVB003 and MV151 preferentially target β5, but eventually label also β2 and β1, whereas RhSylA targets both β2 and β5 and labels β1 only to a low extend.

**Epoxomicin-based probes target β5 in vivo**

MVB003 preferentially labels a 25 kDa protein that we assumed is β5. However, since this signal runs faster compared to MV151 and RhSylA signals, we determined the identity of the proteins labeled **in vivo** by epoxomicin-based probes. We used MVB070 (**Figure 3A**), an azide (N3) minitagged version of MVB003, which causes identical labeling profiles when compared to MVB003 (**Figure 3B**). Cell cultures were labeled with MVB070 and proteins were extracted under denaturing conditions. Azide-labeled proteins were biotinylated with biotin-alkyne using copper-(I)-catalyzed ‘click-chemistry’ reaction (Speers and Cravatt et al., 2004; Kaschani et al., 2009). Biotinylated proteins were purified, separated on gel and the 25 kDa signal (**Figure 3C**) was excised and digested with trypsin (Kaschani et al., 2009). Peptides were analyzed by mass spectrometry and matched to the Arabidopsis protein database. Proteasome subunits PBE1 (At1g13060) and PBE2 (At3g26340) were identified with high coverage and multiple unique peptides (**Figure 3D**). Both PBE1 and PBE2 are β5 catalytic subunits that only differ in a few
amino acids (bold in Figure 3D). These data confirm that the epoxomicin-based probes preferentially label the β5 proteasome catalytic subunit \textit{in vivo}.

Structure-activity relationships of SylA derivatives

SylA contains a 12-membered ring with two double bonds. A natural SylA variant, syringolin B (SylB), differs from SylA by having one of these bonds saturated (Figure 4A). Preincubation of leaf extracts with SylB suppresses MVB072 labeling, but concentrations needed for inhibition are higher when compared to SylA (Figure 4B, lane 7), consistent with the weaker binding of SylB to the yeast proteasome (Clerc et al., 2009b). To verify the importance of the second unsaturated bond, we tested a SylA derivative where this bond is saturated (SylA-sat, Figure 4A). Preincubation with SylA-sat does not prevent proteasome labeling (Figure 4B, lane 6), indicating that the double bond is essential for proteasome inhibition. The importance of this double bond is consistent with the proposed inhibition mechanism because this is the Michael system that is attacked by the catalytic threonine of the proteasome (Groll et al., 2008).

Besides the 12-membered ring, SylA also contains two L-Val amino acids linked through an ureido bond. To test the importance of the conformation of these two valines, stereoisomers were generated and tested. SylA-D-L and SylA-D-D (Figure 4A), which both carry D-Val at the first position (position 1), are able to inhibit β5 but not β1 or β2 (Figure 4B, lanes 4 and 5). In contrast, SylA-L-D which carries a D-Val at the position 2 (Figure 4A) is still effective in proteasome inhibition when compared to the natural SylA (SylA-L-L) (Figure 4B, lane 3), indicating that the conformation of the valine at the position 2 is not important for the selectivity of proteasome inhibition by SylA. Thus, the Michael system is essential for overall reactivity, whereas specificity for inhibition of the β2 subunit requires the L-configuration of the Valine at position 1.

SylA suppresses MVB003 fluorescence mostly in the nucleus

We next used MVB003 to image proteasome labeling of Arabidopsis cell cultures by confocal microscopy. MV151 was not used since this probe also labels PLCPs \textit{in vivo} (Figure 2). Cell cultures were incubated with 2 μM MVB003 and subsequently washed before imaging. MVB003 labeling causes fluorescence in the nucleus and cytoplasm, but not in the vacuole, consistent with the subcellular location of the proteasome (Figure 5A). Pre-incubation with a sublethal dose of SylA and epoxomicin suppresses fluorescence in the nucleus and in the cytoplasm (Figures 5B and 5C, respectively). However, in contrast to epoxomicin, which suppresses overall fluorescence, SylA suppresses mostly nuclear fluorescence. Quantification of the fluorescence over multiple images confirms that MVB003 fluorescence is suppressed upon
pre-incubation with SylA mostly in the nucleus, but also in the cytoplasm (Figure 5E, blue bars). In contrast, preincubation with epoxomicin only moderately suppresses fluorescent signals in the nucleus and cytoplasm (Figure 5E, green bars). Interestingly, these data suggest that SylA suppresses proteasome labeling mostly in the nucleus.

**Accumulation of RhSylA in the nucleus requires the reactive ring system**

To further investigate a potential subcellular targeting by SylA, we used RhSylA for *in vivo* imaging. Importantly, fluorescence caused by RhSylA accumulates in the nucleus, whereas only weak fluorescence is in the cytoplasm (Figure 5F). The RhSylA colocalizes with Hoechst33342, which stains the nuclei of living cells (Figure S2). To determine if the signal depends on the reactivity of SylA, we generated a rhodamine-tagged version of SylA-sat, which lacks the reactive Michael system and is unable to compete with proteasome labeling (Figure 4). Imaging of cells incubated with RhSylA-sat did not show an accumulation of nuclear fluorescence (Figure 5G), suggesting that the nuclear fluorescence observed with RhSylA represents RhSylA that is covalently bound to the proteasome. Furthermore, nuclear fluorescence could be suppressed by preincubation with 50 μM SylA, but not with SylA-sat (Figures 5H and 5I, respectively). Addition of 50 μM SylA after preincubation with 2 μM RhSylA does not suppress proteasome labeling and nuclear fluorescence, indicating that nuclear accumulation of RhSylA is irreversible (data not shown). Analysis of the proteins extracted from the labeled cell cultures confirmed that RhSylA labels the proteasome, and that RhSylA labeling can be suppressed by SylA but not SylA-sat (Figure 5J). Notably, significant amounts of free, unreacted probe were found in the frontier of the protein gel (Figure 5J), indicating that some of the free probe stayed inside the cells after washing. However, since free probe is also present in the absence of nuclear fluorescence (Figure 5H and lane 3 in Figure 5J), the free probe is probably distributed throughout the cells. In conclusion, these data show that RhSylA accumulates in the nucleus. That nuclear accumulation can be prevented by adding and excess SylA before, but not during the labeling, indicates that nuclear RhSylA accumulation is irreversible.

**SylA labels both the nuclear and cytoplasmic proteasome**

To verify the subcellular targeting biochemically, subcellular fractionation experiments were performed. We labeled cell cultures with and without MVB003 and RhSylA and generated nuclei-enriched (NE) and nuclei-depleted (ND) fractions. Subcellular markers PEPC for cytoplasmic proteins and histone H3 for nuclear proteins confirmed that the fractions were not cross-contaminated (Figure 6A). The proteasome subunit PBA1 was detected in
both the ND and NE fraction and the relative signals indicate that only 5% of the cellular proteasomes are localized in the nucleus, given the fact that the NE fraction was 10x concentrated compared to the ND fraction (Figure 6A). Importantly, treatment with MVB003 or RhSylA does not affect the PBA1 levels in the different compartments, indicating that these probes do not influence the subcellular distribution of the proteasome. Analysis of fluorescently labeled proteins reveals that RhSylA labels both β2 and β5 subunits in the nucleus and the cytoplasm, but the signals in the nucleus are relatively stronger (Figure 6A, lanes 5 and 6). Surprisingly, also MVB003 causes relatively strong labeling of the nuclear proteasome (Figure 6A, lane 3), which is in contrast to the strong fluorescence of MVB003 in the cytoplasm (Figure 5).

To investigate if SylA itself targets the nuclear proteasome, we took advantage of the fact that covalent labeling of the β1 subunit causes a shift on the western with anti-PBA1 antibody (Gu et al., 2010). To maximize β1 labeling with SylA, we incubated cell cultures with 50 and 100 μM SylA. Even though the difference in molecular weight (MW) is small, the SylA-β1 conjugate is clearly separated from the unreacted β1 since a signal with a slightly higher MW appears in total extracts (T) of SylA-treated cells (Figure 6B, lanes 1-3). Subcellular fractionation of this sample into a nuclei-depleted (ND) and nuclei-enriched (NE) fraction showed that the SylA-β1 conjugates occur in both the cytoplasmic and nuclear fractions (Figure 6B, lanes 4-9). The ratio of the SylA-β1 when compared to the unreacted β1 is similar between the nuclear and cytoplasmic fractions, indicating that SylA labels the proteasome in both the cytoplasm and nucleus.

DISCUSSION

Through a thorough characterization of in vivo and in vitro profiling and imaging with three unrelated proteasome probes, we have established new procedures for proteasome studies and determined the subunit and subcellular specificity of proteasome inhibitor SylA, a bacterial small molecule effector released by some P. syringae strains during infection.

SylA targets β2 and β5 catalytic subunits of the plant proteasome

We found that SylA preferentially targets only two of the three catalytic subunits of the plant proteasome. RhSylA preferentially labels the β2 and β5 subunits in vitro during short labeling times and at low RhSylA concentrations (Figures 1C and 1D). The same subunit selectivity by RhSylA was observed in vivo (Figures 2C, 2D and 6A). The subunit selectivity does not reside in the reporter tag since we observed that SylA itself preferentially competes with labeling on
β2 and β5, both in vitro (Figure 1E) and in vivo (Figure 2D). The subunit selectivity is different from that of MV151 and MVB003 which preferentially label β5. β1 labeling is slow for all probes, though β1 is best labeled by MVB003 or MV151 (Figure 1C).

The subunit selectivity of SylA was also observed with studies on the yeast proteasome (Groll et al., 2008) and can be explained using the crystal structure of the yeast proteasome inhibited by SylA (Groll et al. 2008, PDB code 2ZCY). The crystal structure of the 20S yeast proteasome contains six SylA molecules, three on each of the two middle rings of β subunits (Figure 7A). SylA is covalently bound to the N-terminal threonine of β1, β2 and β5, and the dipeptide tail of SylA also interacts with the adjacent subunit (Figure 7B). The structure of the adjacent subunits has important implications on how SylA can bind to each of the three binding pockets. Overlay of the SylA structures shows that the dipeptide tail of SylA is pushed downwards when bound to the β1 subunits but not when bound to the β2 and β5 subunits (Figure 7C). This is caused by a bulky H116 side chain in the subunit adjacent to the β1 subunit that makes the β1 binding pocket smaller when compared to that of the β2 and β5 subunits (Figures 7D-F). Consequently, SylA bound to the β1 binding pocket is unable to make a hydrogen bond with D114 of the adjacent subunit, which is an important interaction of SylA bound to the binding pocket of β2 and β5 (Figures 7D-F). The presence of the D114 interaction in β2 and β5 binding pockets explains why SylA preferentially targets the β2 and β5 subunits. Since many properties including H116 and D114 are conserved in the proteasome subunits of Arabidopsis, it seems likely that this interpretation from the yeast crystal structure might also apply for the Arabidopsis proteasome.

We found that the conformation of the valine at position 1 of the dipeptide tail of SylA contributes to the specificity for the β2 subunit since the SylA derivatives carrying a D-Val at this position have a reduced affinity for β2 (Figure 4B). Also this observation can be explained using the crystal structure of the yeast proteasome bound to SylA (Figure 7). The binding cleft of β2 is narrower compared to β5 because it carries glutamine at position 22 (Q22) when compared to alanine (A22) in β5. The side chain of D-Val at position 1 (atom 18 in SylA) would clash with the narrow cleft of β2 (Figure 7E), but not with the wider cleft of β5 (Figure 7F).

Subunit selectivity might be an important aspect of SylA function. Hatsugai et al. (2009) demonstrated that the proteasome mediates the discharge of the vacuolar content into the apoplast during the hypersensitive response, triggered by avirulent P. syringae bacteria. Inhibition studies indicated that this is regulated through the caspase activity of the β1 proteasome subunit (Hatsugai et al., 2009). Consistent with this observation, we found that SylA-producing bacteria are able to prevent early host cell death (Misas-Villamil, Kolodziejek and Van der Hoorn, unpublished), but our data indicate that this is more likely mediated by...
inhibition of the β2 and β5 subunits of the host proteasome. Subunit selectivity is also an important aspect of drug development since proteasome inhibitors are important anti-cancer drugs. Interestingly, SylA was found to induce apoptosis and inhibit cancer proliferation (Coleman et al., 2006) and selectively targets the proteasome, also in cancer cells that have adapted to the proteasome-targeting drug bortezomib (Clerc et al., 2009a).

Nuclear accumulation of SylA

Unexpectedly, imaging experiments show that SylA and RhSylA accumulate in the nucleus. MVB003 causes fluorescence in both the nucleus and cytoplasm, but preincubation with SylA suppresses mostly the nuclear fluorescence, in contrast to preincubation with epoxomicin, which suppresses fluorescence in both compartments (Figure 5A-E). Furthermore, RhSylA causes mostly nuclear fluorescence (Figure 5F). The fact that RhSylA-sat does not accumulate in the nucleus (Figure 5G) and that nuclear fluorescence by RhSylA labeling is not washed out (Figure 5F) or suppressed by adding an excess SylA after RhSylA labeling indicates that RhSylA is immobilized in the nucleus. However, subcellular fractionation studies could not confirm that SylA preferentially labels the nuclear proteasome (Figure 6). There can be many explanations for this apparent discrepancy. One explanation might be that the protocol for subcellular fractionation does not exclude ex vivo labeling because SDS would disintegrate the nuclear compartment before separation. Free, unreacted probes that accumulates in the cells and remain after washing (Figure 5J), might react ex vivo with the proteasome in both the cytoplasmic and nuclear fractions, hiding signals caused by in vivo labeling. However, nuclear fractionations in the presence of proteasome inhibitor MG132 did not reduce labeling of the nuclear proteasome by MVB003 (Figure S3). Another explanation might be that when used at high SylA concentrations, proteasome labeling occurs also in the cytoplasm.

Several mechanistic explanations for potential nuclear targeting of SylA can be excluded by the current dataset. One possibility is that SylA is transported to the nucleus and reacts more efficiently with the nuclear proteasome because the SylA concentration is higher in the nucleus. This would imply that SylA is a cargo for the nuclear import machinery. However, we can rule out the possibility that free SylA concentrates in the nucleus since nuclear fluorescence by MVB003 or RhSylA is comparable by preincubation with SylA but not the inactive SylA-sat derivative (Figure 5). It might be that SylA blocks nuclear import at high concentrations, preventing probes from entering the nucleus. However, SylA was found to react with the nuclear proteasome using subcellular fractionation experiments. A third explanation is that SylA-labeled proteasomes move from the cytoplasm to the nucleus. However, although nuclear import of proteasomes from the cytoplasm is regulated and proteasome inhibition may
increase proteasome import into the nucleus (Takeda and Yanagida, 2005), we did not see an increased PBA1 concentration in the nucleus upon SylA treatment during fractionation experiments (Figure 6). A fourth explanation is that the different reporter tags in MVB003 (bodipy) and RhSylA (rhodamine) have a different fluorescence in the nucleus and cytoplasm. However, such difference in subcellular fluorescence has not been described for these fluorophores before, and we found that also untagged SylA suppresses nuclear fluorescence of MVB003 labeling. A fifth possibility is that the proteasome in the nucleus is more active when compared to the proteasome in the cytoplasm. This would be consistent with the strong labeling of the nuclear proteasome by both MVB003 and RhSylA (Figure 6B). A sixth mechanism for nuclear targeting may be that SylA has a higher affinity for the nuclear proteasome, e.g. mediated by a different composition of the proteasome. Although a different composition of the nuclear proteasome has not yet been described yet, compositions and functions of proteasomes may differ. The immunoproteasome described in animals, for example, appears during immune responses and contains different catalytic subunits that are responsible for the release of more hydrophobic peptides that are used for antigen display (Rock et al., 1994; Goldberg et al., 2002). Differences in proteasome compositions and possible subcellular targeting by SylA are an interesting topic for future studies.

Nuclear accumulation of SylA may have important biological implications. The proteasome degrades a series of nucleus-specific proteins, such as transcriptional regulators. For example, the proteasome degrades nuclear proteins NPR1, EIN2, and JAZ, which are important components of the signaling cascades of the stress hormones salicylic acid, ethylene and jasmonate, respectively (Spoel et al., 2009; Qiao et al., 2009; Thines et al., 2007; Chini et al., 2007). SylA might be produced to interfere in these pathways. Indeed, we found that SylA promotes bacterial growth during SA-induced immunity (Misas-Villamil, Kolodziejek and Van der Hoorn, unpublished results). Interfering with nuclear but not cytoplasmic proteasome activities might therefore be beneficial for Pseudomonas syringae producing SylA.

New tools for in vivo proteasome activity profiling and imaging

During our studies on SylA targeting we made important technological advances to study the proteasome in vivo. Toxicity of the probes during in vivo labeling was found to be at acceptable low levels when low probe concentrations and relatively short labeling times are used, probably because these probes only label a fraction of the active proteasomes. We showed that the epoxyketone-based MVB003 and the syrbaactin-based RhSylA are highly specific proteasome probes, whereas the vinyl-sulfone based MV151 also labels papain-like cysteine proteases, especially in vivo (Gu et al., 2010). These characteristics have important implications for the use
of these probes in vivo. MV151 has the advantage that it displays both proteasome and protease activities in activity profiles, allowing simultaneous monitoring of both proteolytic machinery. This revealed, for example, that the frequently used proteasome inhibitor MG132 preferentially targets PLCPs when used in vivo (Kaschani et al., 2009), which has important implications for the conclusions from studies where protein degradation was studied using MG132. In contrast to in vivo proteasome activity profiling, imaging of proteasome labeling, however, should preferentially be done using MVB003 or RhSylA, since these probes do not label PLCPs. In addition, we found that the labeling of the different subunits depends on the probes used and on timing and probe concentration. Furthermore, we found that proteasome labeling occurs within minutes in vitro and in vivo. Therefore, special care had to be taken to prevent ex vivo labeling during extraction of in vivo labeled materials. Quick in vivo labeling indicates that there is an efficient uptake of these probes through the cell membrane. Thus, our study introduces the parameters and probes to study the proteasome in vivo.

MATERIALS AND METHODS

Chemicals
E-64d and epoxomicin were from Sigma and BioMol, respectively. Synthesis of MV151, SylA, Rh-SylA and other SylA derivatives has been described previously (Verdoes et al., 2006; Clerc et al., 2009a; 2009b, 2010a, 2010b). Synthesis of MVB003, MVB070 and MVB072 have been described in the supplemental document. Aliquots of the probes and inhibitors are available upon request.

Plant materials
Arabidopsis thaliana ecotype Columbia plants were grown in a growth chamber at 24 °C (day)/20 °C (night) under 12-hour light regime. Rosette leaves of 4 to 6-week-old plants were used for protein extraction. Cell cultures (Arabidopsis ecotype Landsberg; May and Leaver, 1993) were weekly subcultured in medium [3% w/v sucrose, 0.5 mg/L naphthalene acetic acid, 0.05 mg/L 6-benzylaminopurine (BAP) and 4.4 g MS Gamborg B5 vitamins (Duchefa), pH 5.7].

In vitro labeling of Arabidopsis leaf extracts
Proteins were extracted by grinding seven rosette leaves into 700 μL water. The extract was cleared by centrifugation (2 min at 16,000g). Labeling was usually done by incubating ~100 μg protein in 50 μl containing 125 mM Tris buffer (pH 7.0), in the presence of 1 μM and 2 μM
probe for 2 hours at room temperature (22-25 °C) in the dark under gentle agitation. Equal volumes of DMSO were added to the no-probe controls. The extract was mixed with 4x SDS-PAGE loading buffer containing β-mercaptoethanol, and separated on 12% SDS gels (~ 10 μg protein per lane). Labeled proteins were visualized by in-gel fluorescence scanning using a Typhoon 8600 scanner (Molecular Dynamics) with excitation and emission at 532 and 580 nm, respectively. Alternatively, the Fuji FLA6000 fluorescence scanner was used. Fluorescent signals were quantified with ImageQuant 5.2 (Molecular Dynamics). After SDS PAGE, proteins were transferred onto polyvinylidene fluoride membrane (Immobilon-P, Millipore) and detected using anti-PBA1 antibodies (1:5,000; BioMol), followed by with HRP-conjugated anti-rabbit secondary antibodies (1:5,000; Amersham). Competition or inhibition assays were done by preincubating the protein extracts with competitor or inhibitor molecules for 30 minutes before labeling with activity-based probes. Time course experiments were done by taking 50 μL aliquots from a 1 mL reaction volume at various time points.

**In vivo labeling and competition**

Cell cultures were diluted by adding 20 μL culture media to 80 μL cell culture and kept at room temperature under gentle shaking in the presence or absence of inhibitors for 30 minutes. Probes were added and labeling continued in the dark for 120 minutes. The cell culture was washed twice with culture medium, and three times with water, and grounded in the presence of 2% SDS. The extract was cleared by centrifugation (2 min. 16.000g) and the 80 μL supernatant was taken, mixed with 4x gel loading buffer and prepared for protein gel electrophoresis. Time course experiments were done by taking 100 μL aliquots from the reaction volume at various time points.

**Nuclear fractionation**

Cell cultures were labeled with 2 μM RhSylA for 2 hours. Cells were washed with Honda buffer [25 g/L Ficoll 400 (Sigma); 50 g/L Dextran T40 (Pharmacia Biotech); 0.4 M sucrose; 25 mM Tris pH 7.4; 10 mM MgCl2; protease inhibitor cocktail (Sigma); and 5 mM DTT] and homogenized in a mortar in 5 mL Honda buffer. The extract was centrifuged gently at 201 g through a 62 μm nylon mesh, and 0.5% Triton-X100 was added to the filtrate. After 15 minute incubation on ice, a 200 μL sample was taken as total extract (T), and mixed with 80 μL gel-loading buffer. The extract was centrifuged (5’ 1500g) and a 200 μL sample was taken from the supernatant as nuclear depleted (ND) extract, and mixed with 80 μL gel-loading buffer. The pellet was washed twice with 3 mL Honda buffer containing 0.1% Triton-X100 and dissolved in 200 μL gel loading buffer, resulting in a nuclear enriched (NE) fraction that is 20-fold more
concentrated than the NE and T fractions. Proteins were separated on protein gels and detected by fluorescent scanning and using antibodies for PBA1, PEPC and Histone, as previously described (Noel et al., 2007; Chang et al., 2009).

Activity-based imaging

The fluorescence of RhSylA (rhodamine ex543/em570), MVB003 (Bodipy ex532/em580), and Heuchst3342 (ex350/em450) was detected by Carl Zeiss LSM 510 confocal microscope. Confocal microscopy was performed with HeNe1 (ex534) laser and the UV laser, respectively. The Zeiss LSM Image Examiner was used for confocal image processing. MVB003 signals were quantified by photometric measurements in situ within 550-600nm ranges. All experiments were done with identical acquisition settings for each probe.

ACKNOWLEDGEMENTS

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Supplemental figures and documents

Supplemental Figure S1 Structures of probes and inhibitors used in this study.
Supplemental Figure S2 Dual labeling of cell cultures with Hoechst and RhSylA
Supplemental Figure S3 Nuclear fractionation in the presence of proteasome inhibitor MG132
Supplemental Document S1 Synthesis of MVB003, MVB070 and MVB072.

LITERATURE CITED


Figure 1 Characterization of three probes for *in vitro* proteasome labeling

A, Inhibitors and probes used in this study. Probes and inhibitors are based on vinyl sulfone (top), epoxomicin (middle); or syringolin A (bottom). The reactive groups are indicated in red. Probes carry a reporter tag at position R (blue).

B, Comparison of *in vitro* labeling with the three probes. Extracts from cell cultures were labeled for two hours with 2 μM MV151, RhSylA or MVB003. Proteins were detected from protein gels by fluorescent scanning and coomassie staining. Dashed lines indicate lanes combined from the same gel.

C, Time course of proteasome labeling. Arabidopsis leaf extracts were labeled with 2 μM MV151, MVB003 or RhSylA and samples were taken at different time points and quenched in SDS sample buffer. Fluorescent proteins were detected by fluorescence scanning. Arrows at the bottom of the gels indicate the probe concentration required for half-maximal labeling.

D, Labeling by SylA at different probe concentrations. Arabidopsis leaf extract was labeled with various concentrations of RhSylA and signals of the different subunits were quantified from protein gels and plotted against the RhSylA concentration.

E, Suppression of proteasome labeling at different SylA concentrations. Arabidopsis leaf extracts were preincubated with various SylA concentrations and labeled with 2 μM MVB072. Signals of the different subunits were quantified from protein gels and plotted against the SylA concentration. Fluorescence was normalized to the no-inhibitor control.

Figure 2 Characterization of *in vivo* proteasome labeling.

A, Viability of cell culture after incubations with probes and inhibitors, measured with Evans blue staining and compared to the heat shock (HS) control. Cell cultures were preincubated for 30 minutes with various inhibitor concentrations and labeled for 2 hours with 2 μM MVB003. Cell death was quantified from Evans blue staining and compared to a heat-shock control (=100% cell death). Error bars represent SEM of three measurements. This experiment was repeated twice with similar results.

B, SDS during extraction prevents *ex vivo* labeling. Lanes 1-4: cell cultures were preincubated with DMSO, washed and proteins were extracted in 2 μM RhSylA containing 0, 1, or 2% SDS (lanes 2, 3, and 4, respectively). Lane 5: cell cultures were preincubated with 2 μM RhSylA and proteins were extracted in 2% SDS.

C, Comparison of *in vitro* and *in vivo* labeling. Cell cultures (c, cells, *in vivo*) or extracts of cell cultures (e, extracts, *in vitro*) were labeled for two hours with 2 μM MV151, RhSylA or MVB003. Proteins were extracted and detected from protein gels by fluorescent scanning and
coomassie stain. Differences in protein amounts are caused by differences in protein extraction efficiency from cell cultures. *, background signal.

D. *In vivo* inhibition confirms target selectivity. Arabidopsis cell cultures were preincubated with 10 μM E-64d or epoxomicin or 50 μM SylA for 30 minutes and then labeled with 2 μM MV151, RhSylA or MVB003 for 2 hours. Proteins were extracted under denaturing conditions, separated on protein gels and detected by fluorescence scanning and coomassie staining.

**Figure 3** Identification of epoxomicin-labeled proteins *in vivo*

A, Structure of minitagged fluorescent epoxomicin derivative MVB070. Red, reactive group; yellow, fluorescent Bodipy group; blue, azide minitag.

B, Comparison of labeling profiles using MVB003 and MVB070. Arabidopsis cell cultures were labeled *in vivo* with MVB003 and MVB070 and fluorescently labeled proteins were detected from protein gels using fluorescence scanning. NPC, no-probe control.

C, Identification of *in vivo* labeled proteins. Cell cultures were labeled with MVB070. Proteins were extracted under denaturing conditions and azide-labeled proteins were biotinylated using ‘click-chemistry’. Biotinylated proteins were purified, separated on protein gels and the fluorescent signal at ~25 kDa was excised, digested with trypsin and identified by mass spectrometry.

D, Identified peptides are underlined in the full-length protein sequences of two different β5 subunits: PBE1 and PBE2. Differences between PBE1 and PBE2 are indicated in bold, the prodomain (first line) is in gray and the N-terminal catalytic threonine is red encircled.

**Figure 4** Structure-activity relationship of SylA derivatives

A, Structures of SylA derivatives. Differences with the naturally occurring SylA-L-L are indicated with circles, and concern double bonds 1 and 2 (red) and stereocenters in Val 1 and 2 (green).

B, Inhibition of labeling by SylA derivatives. Arabidopsis leaf extracts were preincubated for 30 minutes with 100 μM SylA derivatives and then labeled for 2 hours with 1.6 μM MVB072.

**Figure 5** SylA and RhSylA accumulate in the nucleus

A-D, Imaging of cells with MVB003 after preincubation with and without SylA or epoxomicin. Arabidopsis cell cultures were preincubated for 30 minutes with DMSO, 50 μM SylA or 10 μM epoxomicin, and labeled for 2 hours with 2 μM MVB003. Cells were washed and imaged by confocal microscopy.
E, Quantification of fluorescent images. Fluorescent intensities in the nucleus, vacuole and cytoplasm were quantified at ten positions per cell and for ten cells and represented in a box plot.

F-I, Imaging of cells with RhSylA and RhSylA-sat after preincubation with and without SylA or SylA-sat. Arabidopsis cell cultures were preincubated for 30 minutes with DMSO or 50 µM SylA or SylA-sat and labeled for 2 hours with 2 µM RhSylA or RhSylA-sat. Cells were washed and imaged by confocal microscopy.

J, Analysis of labeled proteins and unlabeled probe from cell cultures labeled as described in (F-I). Proteins were extracted from labeled cells in the presence of 2% SDS and analyzed on protein gels. Detection was done using fluorescence scanning and coomassie staining. The sample front is shown since it contains unlabeled probe.

A-D, F-I. v, vacuole; n, nucleus, c, cytoplasm

**Figure 6** RhSylA and SylA label the proteasome in both the nucleus and cytoplasm

A, Nuclear fractionation of MVB003- and RhSylA-labeled cell cultures. Arabidopsis cell cultures were incubated with 2 µM MVB003 or RhSylA or DMSO for two hours. Cells were homogenized and total extracts (T) were separated into nuclei-depleted (ND) and nuclei-enriched (NE) fractions. Fluorescent proteins were detected by in-gel fluorescence scanning, and proteasome subunit PBA1, cytoplasmic marker PEPC, and nuclear marker histone-3 were detected by western blotting on the same samples.

B, Nuclear fractionation of SylA-labeled cell cultures. Arabidopsis cell cultures were incubated with 50 and 100 µM SylA or DMSO for two hours. Cells were homogenized and total extracts (T) were separated into nuclei-depleted (ND) and nuclei-enriched (NE) fractions. Labeling of the β1 subunit was visualized as a shift on the western blot probed with anti-PBA1 antibody. The image of this blot was stretched vertically. A longer exposure is shown for the lane bordered by dashed lines. Antibodies against PEPC and histone-3 were uses as markers for the cytoplasmic and nuclear proteins, respectively.

The NE fractions correspond to 20x more tissue when compared to the T and ND samples.

**Figure 7** Affinities of SylA (-derivatives) explained by crystallographic data

A, Structure of the 20S core protease of the yeast proteasome. The core protease of the proteasome consists of four rings of α and β subunits: the outer rings of each seven α subunits (light grey), and the inner rings of seven β subunits each (grey and colors).
B. One ring of seven β subunits shown from the side of the inner cavity, with three SylA molecules bound to the catalytic subunits: β1 (red), β2 (green), and β5 (blue). Please note that the tail of SylA interacts with the adjacent subunit.

C. Overlay of SylA bound to the three catalytic subunits. Please note the distinct conformation of the tail of SylA bound to β1 (red).

D. SylA bound to β1. SylA (middle, ball-and-stick) is bound to Thr1 (T1) of the β1 subunit (red). Hydrogen bonding between SylA and T21, G47 and S48 exist, but H116 of the adjacent β2 subunit (grey) pushes the tail of SylA downwards, preventing hydrogen bonding with D114 of the adjacent subunit.

E. SylA bound to β2. SylA (middle, ball-and-stick) is bound to Thr1 (T1) of the β2 subunit (green). Hydrogen bonding between SylA and T21, G47, T48 of β2 exist, as well as with D114 of the adjacent β3 subunit. However, the substrate binding cleft is relatively narrow compared to that of β1 and β5, because of the Q22 side chain.

F. SylA bound to β5. SylA (middle, ball-and-stick) is bound to Thr1 (T1) of the β5 subunit (blue). Hydrogen bonding between SylA and T21, G47, G48 of β5 exist, as well as with D114 of the adjacent β6 subunit. The substrate binding cleft is relatively wide compared to that of β2, because of the small A22 side chain.
### A

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**fluorescence**

**anti-PBA1**

**anti-PEPC**

**anti-His3**

### B

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**[μM]**

**anti-PBA1**

**anti-PEPC**

**anti-His3**