Regulation of stomatal aperture by SAUR proteins in *Arabidopsis thaliana*

By

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Approved:

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Abstract

*SMALL AUXIN UP RNA (SAUR)* genes are the largest class of primary auxin (growth hormone) responsive genes in all land plants, many of which are expressed in actively growing plant tissues. This project focuses on the study of four *SAUR* genes in the model plant *Arabidopsis thaliana*. Previous studies have indicated that SAUR63 promotes elongation of growing cells by regulating other proteins at the plasma membrane. Overexpression of the protein in a broader domain, including in guard cells, caused the guard cells to swell, leading to constitutive stomatal opening. To test whether other *SAUR* genes regulate guard cell function in wild-type plants, we are studying three additional members of the family, *SAUR30*, *SAUR56* and *SAUR60*, which are expressed preferentially in guard cells. We hypothesize that these *SAUR* genes regulate guard cell function based on their localization. We found that plants that overexpressed and stabilized SAUR60 or SAUR63 proteins exhibited constitutively open stomata, even during physiological conditions favoring stomatal closure. These results suggest that SAUR proteins indeed regulate guard cell function. This is the first demonstration that certain members of the SAUR protein family can lead to open stomata. Similar experiments for *SAUR30* and *SAUR56* are in progress. We are currently knocking-out these genes to test if they are required to open stomata. Understanding how stomatal aperture is regulated may lead to improvements in physiological traits affecting photosynthesis and drought resistance in crops.
Introduction

*SMALL AUXIN UP RNA (SAUR)* genes are expressed in actively growing plant tissues and are the largest class of primary auxin responsive genes in all land plants (Ren and Gray, 2015). These genes are members of a large gene family, many of which are induced by the growth hormone auxin. To study this protein family, we have chosen to work with the model organism *Arabidopsis thaliana*. In growing *A. thaliana* hypocotyls, 26 out of 49 genes induced within 30 minutes of auxin application are SAUR genes (Chapman *et al.*, 2012). Several SAUR proteins, including SAUR19 and SAUR63 are found to localize to the plasma membrane and promote growth (Chae *et al.*, 2012, Spartz *et al.*, 2012). Among these, SAUR19 can inhibit phosphatases that inactivate the plasma membrane H⁺-ATPase (Spartz *et al.*, 2014). Other SAUR proteins may act similarly, but it is also possible that they have distinct biochemical functions.

The SAUR63 gene promotes cell growth by regulating other proteins at the plasma membrane (Chae *et al.*, 2012). We found that overexpression of the protein throughout the plant, including in guard cells, caused the guard cells to swell, leading to constitutively open stomata. Guard cells are important for plants because they swell and shrink in order to regulate gas exchange through the stomata on the leaf surface. To test whether other SAUR genes regulate guard cell function in wild-type plants, we focused on three additional members of the family, *SAUR30, SAUR56* and *SAUR60*, which have been found to be expressed preferentially in guard cells (Bauer *et al.*, 2013). Further studies on the previously characterized *SAUR63* were also carried out to test whether SAUR proteins are sufficient in causing stomatal phenotypes. Therefore, we hypothesize that these SAUR genes regulate guard cell function. We predict that
SAUR56 and SAUR60 will have similar functions since they are sister genes. On the other hand, SAUR30 has a more distinct sequence and is also known to have an opposite regulation; SAUR56 and SAUR60 are repressed by drought or the stress hormone ABA, whereas SAUR30 is induced by drought and ABA. (Bauer et al., 2013)

The overarching project aims to determine the role that SAUR proteins have in regulating physiological responses (guard cell swelling) indirectly involved in processes impacting cell growth, such as photosynthesis and respiration. Future goals include identifying the regulatory targets of each SAUR protein as well as elucidating the structural requirements for their localization and function. Understanding how stomatal opening is regulated could possibly improve understanding of, and modifications to, traits directly involved in photosynthesis and drought resistance in crops.

**Methods**

**Generation of transgenic plants**

Vectors containing the gene of interest with detectable protein tags were constructed and cloned into Agrobacterium tumefaciens strain GV3101 via electroporation. Table 1 provides a summary of the vectors and primers used as well as the gene constructs we made. The HA tag was selected due its small size so that the fusion protein can maintain its native configuration, although these lines have not been characterized yet. Gus fusion proteins can easily be detected by staining to determine which cells express the fusion, whilst GFP tags can be viewed via confocal fluorescence microscopy to determine subcellular localization.
The genes of interest were amplified using gene-specific primers and cloned into Gateway binary vectors (Table 1). These constructs were transfected into A. tumefaciens and then introduced into wild-type A. thaliana (ecotype Columbia) plants by floral dip (Clough & Bent, 1998). The successfully transformed plants were then selected on MS-agar (Murashige and Skoog salts) medium containing 30 µg ml\(^{-1}\) kanamycin: the first generation of plants was screened for single-locus insertions by 3:1 segregation in kanamycin resistance. Multiple subsequent generations of plants were then grown and harvested to select for the homozygote containing the gene of interest.

**Fluorescence imaging for subcellular protein localization**

Homozygous seedlings containing the **SAUR60**:GFP gene were analyzed by taking images on a Zeiss 710 DUO confocal microscope. A negative control of wild-type seedlings was used as a baseline to deduce localization of the protein.

**Gus-staining imaging for cellular protein localization**

Homozygous seedlings containing the **SAUR56**:Gus gene were stained with staining solution (50 mM NAHPO\(_4\), 5 µg ml\(^{-1}\) of X-gal, 1.75 mM of ferricyanide, 1.75 mM of ferrocyanide and 0.1% Triton-X buffers) and analyzed by taking images on a compound microscope. A negative control of wild-type seedlings and a positive control of plants known to exhibit staining in the presence of X-gal, were used as baselines to deduce localization of the protein.
Hypocotyl Length Measurement

Seeds of respective genotypes were plated and grown vertically for 4-5 days in 0.5x MS, 0.6% phyto-agar plates. We then took photographs of the seedlings to measure the hypocotyl length of each seedling using ImageJ (Schneider et al., 2012). Measured lengths were averaged and a Student’s t-test for significance was performed using Microsoft Excel.

Stomatal Aperture Assays

Lower (abaxial) epidermal cells from leaves were mounted onto a slide using medical adhesive glue, incubated in opening buffer (10 mM KCl, 10 mM MES, pH 6.15) for 1 hour and stained with Toluidine Blue to be imaged. We measured responsiveness to different light levels, and ABA concentration. An initial experiment tested for response to the absence and presence of each variable (light and ABA) before dosage response experiments were carried out to determine responsiveness to varied levels of light or ABA concentration. To determine the effect of light, lower epidermal cell samples were incubated in darkness, intermediate or high light levels (0 µmol m$^{-2}$ s$^{-1}$, 15.50 µmol m$^{-2}$ s$^{-1}$ and 157.20 µmol m$^{-2}$ s$^{-1}$ respectively) for 2 hours before being imaged. In the experiment testing the effect of varied ABA concentration, once the 2 hour long incubation in opening buffer was completed, some tissue samples were incubated in 0 µM, 1 µM, 10 µM or 100 µM ABA solutions (in opening buffer) for 30 minutes before being imaged. Stomatal aperture ratios were measured with ImageJ software (Schneider et al., 2012) and analyzed quantitatively as the ratio of stomatal opening divided by stomatal length. The stomatal aperture ratios were averaged and analyzed with a Two-way ANOVA, followed by Tukey’s multiple comparison test for significance against each sample mean.
Furthermore, a leaf drying assay was conducted as an indirect measure of stomatal aperture. Leaves from respective genotypes were detached and weighed. The leaves were then placed under bright light (157.20 µmol m\(^{-2}\) s\(^{-1}\)) to allow for water loss over a span of 120 minutes. Measurements were taken after 30, 60 and 120 minutes, and the collected data were normalized to initial weights and analyzed using standard t-tests for significant difference in water loss. Lastly, the circadian rhythm of stomatal opening was studied indirectly, via measurement of stomatal apertures of transgenic plants both 2 hours before the lights in the growth chambers came on and 6 hours after the lights went out. These were times where stomata are expected to be closed according to the natural circadian rhythm of plants.

**Results**

**Overexpressed SAUR60 proteins localized throughout the plant**

Expression of fusion proteins in a larger domain via the \(P_{35S}\) promoter resulted in the expression of SAUR60:GFP proteins throughout the plants, including the roots, hypocotyls and guard cells (Figure 1). The protein was also visualized to localize to the cell membrane, cytoplasm as well as nucleus at a sub-cellular level.

**SAUR56 was confirmed to be expressed in guard cells**

Through Gus staining, it was found that the PSAUR56:SAUR56:Gus protein was expressed in the guard cells (Figure 2). Since the native promoter was used, it can be concluded that the wild-type protein localizes in the guard cell, in agreement with microarray data (Bauer *et al.*, 2012) that indicated preferential expression of *SAUR56* genes in guard cells.
**Overexpressed SAUR63, SAUR60 and SAUR30 affect hypocotyl elongation**

Previously, we found that SAUR63:GFP fusion proteins have a gain-of-function, which results in faster elongation of hypocotyls and flower organs (Chae et al. 2012). Similarly, we found that plants expressing a SAUR60:GFP fusion protein behind a strong viral promoter also have increased hypocotyl elongation, suggesting that SAUR60 has similar activity compared to SAUR63. Furthermore, overexpressed SAUR63 (P₃₅S:SAUR63:YFP:HA, P₃₅S:SAUR63:GUS #9, P₃₅S:SAUR63:GUS #15) and SAUR60 lines (Col x P₃₅S:SAUR60:GFP, P₃₅S:SAUR60), had significantly longer hypocotyls than the wild-type A. thaliana (Columbia ecotype) (Figure 3A). The P₃₅S:SAUR63:YFP:HA transgenic plant had the longest hypocotyls suggesting the strongest expression of SAUR63 proteins or greatest abundance or activity of the protein of interest. Interestingly, the P₃₅S:SAUR63:Gus #9 construct did not show the elongated hypocotyl phenotype likely due to a technical problem in the experiment, as we have observed long hypocotyls in this line in previous experiments. Ost2-2 is a hyperactive form of the H⁺-ATPase that may be a target of SAUR proteins (Merlot et al., 2007), and the ost2-2 mutant also had a long hypocotyl (Figure 3A). Conversely PP2C-D1 belongs to a family of phosphatases that inhibit the activity of H⁺-ATPase (Spartz et al., 2014), and plants overexpressing this gene had short hypocotyls.

In the investigation of the SAUR60:GFP protein, the amount of protein expressed past a certain threshold did not seem to contribute significantly to the elongated hypocotyl phenotype since the heterozygotes and homozygotes exhibited similar elongated hypocotyl lengths (Figure 3B) in relation to the wild-type.
Similarly, hypocotyl lengths were measured in three overexpressed SAUR30 lines. Using the same controls as previously discussed, it was found that the lines, with overexpressed SAUR30, SAUR30:GFP and GFP:SAUR30, showed a trend towards significantly longer hypocotyls (Figure 3H). The overexpressed SAUR30 protein demonstrated the longest hypocotyls, similar to $P_{35S}$:SAUR63:YFP:HA control line, whilst the overexpressed fusion proteins, SAUR30:GFP and GFP:SAUR30, had lengths closer to the intermediate phenotype of the $P_{35S}$:SAUR60:Gus #9 line, suggesting that SAUR30 proteins had similar activities to SAUR60 and SAUR63.

**Overexpressed SAUR60:GFP adversely affects development of the plant**

Despite having largely similar lengthened hypocotyl, the homozygous and hemizygous $P_{35S}$:SAUR60:GFP adult plants had drastically different phenotypes. The homozygous plant was severely dwarfed in comparison to both the hemizygote and wild type plant (Figure 3C). Although the adult homozygote is able to survive, it does not grow significantly larger and appears to be virtually sterile, with the production of only 2 seeds. (Figure 3F, 3G). In contrast, the hemizygote produced viable seeds. However, the hemizygote was also dwarfed, had twisty inflorescences, and thinner, shorter leaves (Figure 3D). These were distinct phenotypes in comparison to the wild-type plant.

**Overexpressed SAUR63 and SAUR60 causes increased rate of water loss**

We found that the overexpressed SAUR63:YFP:HA protein caused the most rapid decrease in leaf weights due to water loss over a span of 120 minutes. This was almost as fast as the control line ost2-2 which is known to have opened stomata phenotypes (Merlot et al., 2007). Hemizygous $P_{35S}$:SAUR60:GFP and homozygous $P_{35S}$:SAUR63:Gus lines also exhibited an
increased rate of water loss but to a smaller degree (Figure 4A). Similar results were observed in two other replications to support its accuracy.

**SAUR60 and SAUR63 proteins led to guard cell phenotypes and affected stomatal aperture**

All the transgenic plants exhibited a higher stomatal aperture ratio in comparison to the wild-type. The overexpressed SAUR63:YFP:HA protein exhibited the largest stomatal aperture ratios, while the hemizygous $P_{35S}$:SAUR60:GFP and homozygous $P_{35S}$:SAUR63:Gus lines had the smallest increase in stomatal aperture size. The $P_{35S}$:PP2-CD1 line also had unexpectedly large stomatal aperture sizes despite exhibiting a wild-type rate of water loss in the water loss assay. (Figure 4C,D) Additionally, the guard cells in the $P_{35S}$:SAUR63:YFP:HA and $P_{35S}$:SAUR60:Gus lines were often observed to be deformed and elongated in comparison to the wild type (Figure 4B).

**Varied light conditions and ABA concentrations changes the stomatal ratios**

Varying the light levels during incubation time altered the stomatal aperture ratios of each genotype to differing degrees (Figure 4B). $P_{35S}$:PP2C-CD1, ost 2-2 and $P_{35S}$:SAUR63:YFP:HA constructs were not significantly affected by decreasing light intensity. However, the two other transgenic plants exhibited more wild-type phenotypes in terms of stomatal aperture size at high light levels. (Figure 4C.1) However, all the genotypes studied were less responsive to decreasing light levels than wild-type. (Figure 4C.2) Lastly, measurement of stomatal aperture 2 hours before the lights came on and 6 hours after the lights went out showed that stomata of $P_{35S}$:SAUR63:YFP:HA lines were constitutively open while wild-type stomata were closed; this was expected according to circadian rhythm of the plant.
Based on our data, ABA (drought-stress hormone) caused wild-type stomata to close as expected. Columbia and \( P_{35S}:PP2C-D1 \) all exhibited closure of stomata in the presence of ABA, while Columbia x \( P_{35S}:SAUR60:GFP \) exhibited closure of stomata to a lesser degree. \( P_{35S}:PP2C-D1 \) was the most sensitive to the presence of ABA, having mostly open stomata in the absence of ABA, and behaved like the wild-type in the presence of ABA. On the other hand, \( ost2-2 \) and \( P_{35S}:SAUR63:YFP:HA \) did not appear to be sensitive to the presence of ABA. (Figure 4D.1) Comparable results were yielded from repeating the experiment, suggesting that the activities of SAUR60 and SAUR63 may be interestingly different in some aspects. Similar to light response, all the genotypes studied were less responsive to varied ABA concentration than wild-type. (Figure 4C.2)

**Mutation in aha1 suppressed the effects of overexpressed SAUR63 proteins**

AHA genes encode plasma membrane proton pumps which control guard cell swelling and hence these genes regulate stomata opening (Spartz et al., 2014). It was found that the null mutant \( aha1-6 \) had more closed stomata in both repeats of the experiment. Furthermore, in the double mutant, \( aha1-6 \times SAUR63:YFP:HA \), stomata remained closed in a similar manner to the single mutant. This suggested that mutations in \( aha1 \) gene caused the suppression of effects due to overexpressed SAUR63. However, further experiments have to be carried out to determine whether this was due to silencing of \( SAUR63 \) expression or truly due to suppression by \( aha1 \) mutation. Lastly, we also found that phenotypes of \( aha2 \) single and double mutants were not significantly different from their respective controls (Figure 4E).
Discussion

Our study showed that the overexpression SAUR proteins does indeed have large phenotypic impacts on *A. thaliana*, and these results provide the foundation for further studies into characterizing this protein family. The hypocotyl length studies are a convenient quantitative measure of the effects of SAUR proteins on the elongation growth of *A. thaliana*. It was found that SAUR60:GFP localizes to the plasma membrane and increases elongation, suggesting that its activity is similar to SAUR63 (Chae *et al.*, 2012). Similarly, overexpressed SAUR30:GFP and GFP:SAUR30 also led to elongated hypocotyls, despite SAUR30 expression being known to be induced by ABA, opposite to the regulation of SAUR56 and SAUR60 (Bauer *et al.*, 2012). Further studies such as inflorescence stem growth, flower organ development and fruit sizes should be carried out for a more complete indicator of organ elongation due to SAUR60 and SAUR30 expression. Few or no seeds were harvested from the homozygous *P*:SAUR60:GFP plant and whether or not the plant is truly sterile will be determined in subsequent studies.

Previous studies have demonstrated that, SAUR63 is known to stimulate cell expansion (Chae *et al.*, 2012). Mature guard cells do not grow, but instead they swell reversibly to cause stomatal opening. Thus the stomatal phenotypes caused by increased SAUR63 activity may reveal a similar effect in stomata as in growing organs, since the growth and swelling of cells are presumably caused by similar underlying biochemistry. The results obtained, together with published data (Bauer *et al.*, 2012) that SAUR60 is expressed in guard cells, suggesting that SAUR60 normally regulates stomatal opening.
Comparing the results obtained in the water loss assay and stomatal aperture assay, it was found that the overexpressed SAUR63:YFP:HA construct caused the widest open stomata and the fastest water loss, which is consistent with the previous results demonstrating this line having the strongest hypocotyl phenotype. The SAUR60:GFP plants also had more modest stomatal phenotypes, comparable to their hypocotyl phenotypes. The corresponding results suggest that varying phenotypic strength was likely due to the level of expression of the respective SAUR protein. Furthermore, overexpressed SAUR63:YFP:HA demonstrated constitutively open stomata even at night, showing disruption of circadian rhythm in stomata opening regulation. Similar experiments will be carried out to study the effect of SAUR30, SAUR56 and SAUR60 proteins on the diurnal rhythm of stomatal apertures.

The suppression of stomata opening by aha1 mutations suggested that SAUR proteins act upstream to the AHA1 plasma membrane proton pump. Further studies of other proteins involved in the pathway will determine whether SAUR genes are indeed directly regulating the proton pump.

The PP2C-D1 construct was included since it was found to act in an opposite manner compared to SAUR proteins (Spartz et al., 2014). While results under varied light levels indicated that it had more open stomata than wild-type, it was later found that it was significantly sensitive to the presence of ABA. This suggested that PP2C-D1 proteins might control the stomatal aperture opening mechanism through a different pathway from the SAUR proteins. However, further studies of protein-protein interaction as well as elucidating the
exact upstream and downstream members in the pathway would have to be done to confirm this hypothesis.

Our study is the first to demonstrate that particular members of the SAUR protein family lead to an open stomata. Similar experiments for SAUR30 and SAUR56 are in progress. Further studies are being designed to determine whether near-constitutive stomatal opening in our mutants are due to anomalous growth of the guard cells or regulation by SAUR gene expression. In the next experimental phase, we hope to determine the specific function of SAUR proteins when being expressed in their normal domains, driven by their native promoters. Protein abundance would be studied using the transgenic plants designed with native promoters. Ultimately, our goal is to identify the regulatory targets of each SAUR protein as well as defining the structural requirements for their localization and function. This would involve the purification of the proteins of interest with their interacting partners so that the regulatory targets of each protein can be analyzed. Functional domains and interacting sites can also be identified via proteomic analysis. The identification of knockout mutations in these genes via genome engineering may provide support for the findings of this project.

Through the study of stomatal aperture regulation, improvements in physiological traits affecting photosynthesis and drought resistance may be established to improve crop persistence and ultimately crop yield.
**Figures and Tables**

*Generation of transgenic plants*

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<thead>
<tr>
<th>Gene of interest</th>
<th>Primers</th>
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Table 1: Summary of all gene constructs created for study, with corresponding primers and vectors (Nakagawa *et al.*, 2007) used. Fusion proteins with specific protein tags were constructed behind the native *A. thaliana* promoters and overexpressed viral promoter.

* indicates lines made by Bryan Wang, the other lines were made by Punita Nagapal.
Fluorescence and Gus-staining imaging for protein localization

Figure 1: Representative images showing confocal imaging of overexpressed SAUR60:GFP localization in (A) hypocotyls, (B) roots, and (C) cotyledon. (The red fluorescence indicates the plasma membrane stained with FM4-64 while green indicates GFP. Green fluorescence indicates that the SAUR60:GFP protein localized both in plant cell membrane, cytoplasm as well as the nucleus of cells. Figure 1A was taken by Punita Nagapal.

Figure 2: Representative images showing X-Gluc staining SAUR56:Gus localization in guard cells at (A) 10x and (B) 40x magnifications.
**Hypocotyl measurements and plant growth of SAUR60 and SAUR63 transgenic plants.**

**Figure 3A:** Hypocotyl lengths of seedlings of the indicated genotypes grown for 5 days on 0.5X MS plates. Data are shown as means + SDs; * P < 0.05 in a Students t-test compared with the wild type.

**Figure 3B:** Hypocotyl lengths of seedlings of segregating $P_{35S}$:SAUR60:GFP grown for 5 days on 0.5% MS. Data are shown as means + SDs; * P < 0.05 in a Students t-test compared with the wild type.
Figure 3C-E: Phenotype of 5.5 weeks old segregating transgenic plants as labeled with the wild type segregant on the right of (C), while white arrow in upper left of (C) indicates the dwarfed homozygous plants and left of (C) and (D) show phenotypes of the hemizygote plant. (E) Close up of the homozygous transgenic plant.

Figure 3F-G: Phenotype of 5.5 months old homozygous transgenic plants.
Figure 3H: Hypocotyl lengths of seedlings of the indicated genotypes grown for 5 days on 0.5X MS. Each bar shows results for an independent transgenic line. Data are shown as means + SDs; * P < 0.05 in a Students t-test compared with the wild type.
Water loss and stomatal aperture assay results.

Figure 4A: Representative data of leaf water loss over a period of 120 mins.

Figure 4B: Data of stomatal formation phenotype: (B.1) Data of measured stomata lengths in each genotype. Data are shown as means + SDs; * P < 0.05 in a Students t-test compared with the wild type, (B.2) Representative picture of deformed stomata in $P_{35S:SAUR63:YFP:HA}$ lines in comparison to (B.3) wild-type stomata.
Figure 4C: (C.1) Stomatal Aperture Ratio measurements of indicated genotypes in: varied light condition, only $P_{35}\text{:SAUR63:GUS}$ #9 and Columbia x $P_{35}\text{:SAUR60:GFP}$ exhibit significant differences between low and high light ($P \leq 0.05$). However at low light, these genotypes appeared to be similar to wild-type ($P > 0.05$). (C.2) Results from darkness response where samples were subjected to 3 different light conditions. Two-way ANOVA, followed by Tukey’s multiple comparison test, was used for statistical analysis ($P \leq 0.05$). Means with the same letter are not significantly different from each other.
Figure 4D: (D.1) Stomatal Aperture Ratio measurements of indicated genotypes in: presence of ABA, Columbia, $P_{35S}$:PP2C-D1 and Columbia x $P_{35S}$:SAUR60:GFP all exhibit significant differences in the presence of ABA (P≤0.05). $P_{35S}$:PP2C-D1 exhibit significant difference in the absence of ABA, but behaves like wild-type in the presence of ABA. ost2-2 and $P_{35S}$:SAUR63:YFP:HA exhibit significance difference from wild-type (P≤0.05). (D.2) Results from ABA dose response experiment where samples were subjected to 4 different concentrations of ABA. Two-way ANOVA, followed by Tukey’s multiple comparison test, was used for statistical analysis (P≤0.05). Means with the same letter are not significantly different from each other.
**Figure 4E:** Stomatal Aperture Ratio measurements of indicated genotypes, including *aha* mutants to establish downstream or upstream relations between SAUR proteins and *aha* protein interactions. Data are shown as means + SDs; * P < 0.05 in a Students t-test compared with the wild type.

**Figure 4F:** (F.1-F.3) Representative pictures of a PP2C-D1 transgenic plant lower epidermis photographed showing the same stoma in varying magnifications. (F.4) Picture of a stoma of the same genotype incubated in ABA for 1 hour.
References


a plasma membrane H(+)−ATPase prevents abscisic acid-mediated stomatal closure.

EMBO J., 26(13): 3216-3226.

