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Characterization of drought tolerance in *Arabidopsis* mutant *fry1-6*

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Summary

Drought resistance is a beneficial trait for plants, especially crops, as it allows survival in conditions of low water. Current environmental trends point toward an increased occurrence of drought, while the increasing world population requires more food production. Therefore, drought resistance is a desirable trait in crops. *Arabidopsis thaliana* mutant *fiery1* (*fry1-1* and *alx8*) was previously reported to be drought resistant. In this study, we tested and confirmed that a different mutant allele, *fry1-6*, also exhibited drought resistance capabilities and survived longer than wild-type plants when watering ceases. We sought to discover the cause of the drought resistance of the *fry1-6* mutant. To this end, we compared differences between wild-type and *fry1-6* plants in the transpiration rate under simulated drought conditions, number of stomata per unit leaf area, rate of water loss from cut-off leaves, and water content within soil. Our results revealed that there are no significant differences in those traits, except that *fry1-6* plants withstand drier soil conditions than wild-type plants. Overall, our data suggest that the number of stomata and the transpiration rate are not the primary reasons for the drought resistance of *fry1-6* plants.

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Introduction

According to a recent report on climate change, dangers of climate change have become increasingly clear, and the resulting extreme weather conditions are expected to increase in frequency and intensity (1). Such extreme weather conditions include excessive high or low temperatures, UV irradiation, higher than normal light intensity, and drought, which can compromise crop yield significantly. Among them, prolonged drought conditions are particularly detrimental to agricultural productivity. Water deficit can cause crop harvests to fall below half of their potential yield (2-4), leading to food insecurity, price inflation, and famine. In particular, it will become more difficult to produce enough food to meet the demands of a growing global population under drought conditions resulting from climate change. Because of this, drought resistance is becoming an increasingly important trait for crop plants.

Tremendous effort has been made to modify plants for increased drought tolerance. To this end, various approaches have been used, including conventional breeding and engineering of crop strains, as well as screening of mutants for those with drought-resistant traits. One approach, using reverse genetics, seeks to modify the expression levels of specific target genes (5). Genes modified by this technique are known to be involved in the drought responses, such as signaling, transcriptional control, protection of membranes and proteins, and toxic compound scavenging (6). On the other hand, forward genetic approaches, such as random mutagenesis, have been successfully used to generate mutations in previously uncharacterized genes to produce plants with desirable traits (7).

Arabidopsis SAL1 was originally discovered as a gene that enhances salt tolerance (8). Later, multiple labs have discovered it through independent mutant screening experiments for various developmental and physiological phenotypes, including enhanced cold and osmotic stress response (9,11), leaf shape and venation patterns (12), polar auxin transport (13), sulfur metabolism (14), photomorphogenesis and flowering time regulation (15), lateral root formation (16), and drought resistance (17). Such independent discoveries lead to the usage of various names assigned to the same gene such as *FIERY1 (FRY1)* (9), *ALX8* (10), *HOS2* (11), *ROTUNDA1 (RON1)* (12), *SUPO1* (13). For simplicity, the gene name *FRY1* will be used in this report hereafter.

FRY1 protein is a bifunctional enzyme that possesses both 3'(2'),5'-bisphosphate nucleotidase activity and inositol polyphosphate 1-phosphatase activity (8). Since FRY1 inositol polyphosphate 1-phosphatase activity dephosphorylates inositol biphosphate (IP2), an intermediate in the inositol triphosphate (IP3) degradation pathway, the *fry1* mutant accumulates IP3, which affects Ca^{2+} signal-related biological processes (13). On the other hand, since the nucleotidase activity breaks down the sulfation byproduct 3'-phosphoadenosine-5'-phosphate (PAP) to AMP and inorganic phosphate, the *fry1* mutant accumulates PAP (18). In turn, PAP inhibits exoribonucleases (XRNs) that degrade miRNAs and aberrant RNAs that mediate gene silencing (19). Hence, the above-mentioned pleiotropic phenotypes in *fry1* mutants may be mediated through enhanced gene silencing and/or modified Ca^{2+} signaling.

The drought tolerance phenotype was observed in *alx8* and in *fry1-1* allelic background. These are originated from different *Arabidopsis* variants called ecotypes *Columbia* for *alx8* and *C24* for *fry1-1* (17). Soil-grown *alx8* and *fry1-1* could survive longer than wild-type plants under drought conditions. Moreover, *alx8* exhibited higher relative water content than the wild-type plants after being exposed to prolonged drought conditions (17), but the cause of the higher relative water content was elusive.

In the present study, we tested the drought tolerance of another *fry1* allele, *fry1-6*, which was initially discovered due to its photomorphogenic phenotype (15). In contrast to *fry1-1*, the ecotype background of *fry1-6* is *Columbia*, and *fry1-6* is a knockout mutant induced by a T-DNA insertion in *FRY1* gene, whereas *fry1-1* and *alx8* are base substitution mutants. We hypothesized that *fry1-6* mutant is also drought tolerant, and that the tolerance is due to a lower transpiration rate since transpiration through stomata is the major source of water loss. To test this we measured transpiration rate, leaf water loss rate, soil water content, and the number of stomata. Although our data confirms the drought resistance of *fry1-6*, we did not observe differences in the numbers or transpiration efficiency of stomata.

Results

Drought resistant *fry1* plants have similar transpiration rates as wild-type plants.

Since it has been reported that two different alleles of *fry1* in different ecotype backgrounds (*fry1-1* and *alx8*) could tolerate drought more efficiently (17), we tested another allele *fry1-6* (ecotype *Columbia*) under our laboratory conditions (**Figure 1**). As in other drought tolerant allelic backgrounds, *fry1-6* plants survived longer than the wild-type plants when watering was paused, and the soil was allowed to dry. Since water loss from plants occurs mainly through transpiration at the stomata (20), we hypothesized that a lower transpiration rate was the reason for drought tolerance of *fry1-6* mutants. To measure the transpiration rate, homemade transpirometers were constructed (**Figure 2A**), as described in Methods. The rate of transpiration was measured by weighing the whole transpirometer setup periodically. Loss of weight could only be credited to transpiration, since water could not have evaporated through the oil layer on top of the transpirometer solution, and we did not add or remove liquid from the transpirometer. In fact, we confirmed this by using a control transpirometer without a plant, which showed no detectable change in weight over the same time period we tested (**Figure 2B**).

In addition to normal conditions, simulated drought conditions (hypertonic solutions) were created by using poly[ethylene glycol] solutions (PEG) in the transpirometer (21). In both *fry1-6* and wild-type plants, increasing the concentrations of PEG reduced the average transpiration rate per plant, indicating stomatal closure (**Figure 2C**). Moreover, the transpiration rate for *fry1-6* mutants was significantly lower than that of wild-type plants for every concentration of PEG we used (**Figure 2C**), suggesting that *fry1-6* plants generally conserved water in any level of drought tested in this study. However, *fry1-6* mutant plants were noticeably smaller in size than wild-type plants (**Figure 1**). As a result, it was expected that average transpiration rates per plant would be lower for *fry1-6* plants. To prevent size differences from interfering with the experiment conclusions, transpiration rates per unit area (cm^2) were calculated after obtaining the surface area of the leaves of each individual plant we used. As shown in **Figure 2D**, *fry1-6* plants did not have a lower average transpiration rate per unit area compared to wild-type plants. In fact, their transpiration rates were even slightly higher than the rates in wild-type plants, yet statistically insignificant

Figure 1: Drought resistance of *fry1-6*. Water was withheld for 12 days before the pictures were taken. Wild-type plants were all dried, whereas *fry1-6* plants still survived. Representative pictures of three-week-old plants were shown. Other 4 pots for each genotype showed the same phenotype.

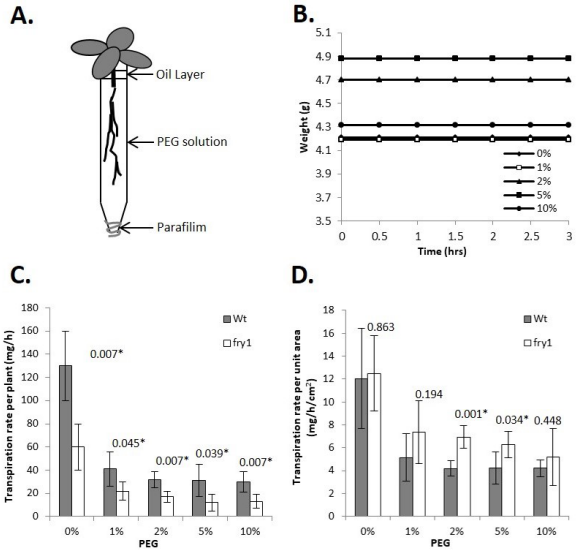

Figure 2: Transpiration rate per plant. (A) Diagram of transpirometer used in this study. **(B)** The actual weight change of the control transpirometers without plants in them. No change was detected. **(C)** Transpiration rate per plant in mg per hour. **(D)** Transpiration rate per unit area (mg/cm^2) per hour. The error bars represent standard deviations (n=5) and the digits above the bars indicate p-values from *Student's* t-tests between wild-type and *fry1-6* plants.

except in 2% and 5% PEG ($p < 0.05$ for both). Despite the obvious drought tolerance in *fry1-6* mutant (**Figure 1)**, our results revealed that they transpired similarly (0%, 1%, 10% PEG) or slightly more (2%, 5% PEG) than wild-type plants during drought conditions when average rates per unit area were calculated. This hints that the drought resistance of *fry1-6* plants is not due to lower transpiration rate per unit area.

Wild-type and *fry1-6* plants have similar numbers of stomata.

Since transpiration occurs through the stomata in leaves, the average numbers of stomata per unit area on *fry1-6* and on wild-type plants were quantified to find out if the transpiration rate data correlate with the numbers of stomata. Using a light microscope, we acquired photographs of stomata imprints in nail polish that was applied to the underside of leaves, and the number of stomata was subsequently quantified using the ImageJ program (22). These data showed that the number of stomata in a single field of view at 200x was not significantly different between the two types of plants ($p = 0.437$; **Figure 3B**). This implies that the *fry1-6* mutant has a similar number of stomata to wild-type plants, and that the transpiration rate for each stoma (i.e. stomatal conductance) is also similar to, or slightly higher than, in wild-type plants (**Figure 2D**).

Leaf water loss rates in wild-type and *fry1-6* plants.

The actual transpiration rate is affected by both the rate of water intake from the root and the rate of water loss through stomata in the leaves. In order to test the rate of water loss from the leaves only, wild-type and *fry1-6* plants' aerial parts (i.e. entire above-ground parts) were separated from their roots and placed on dry plastic trays (**Figure 4A**). The weight of the aerial parts was measured every hour. Weight change could be credited to loss of water mainly through stomata due to evaporation (20). The data revealed that about the same rate of water loss (% of original weight) was detected from the aerial parts of wild-type and *fry1-6* plants during the seven-hour experiment (**Figure 4B**). The small differences in the rate of water loss were statistically insignificant (**Figure 4C**), suggesting that the average rate of water loss per hour is very similar in both wild-type and *fry1-6* plants. This result indicates that the aerial parts of *fry1-6* and wild-type plants do not lose water at significantly different rates, and further implies that *fry1-6* mutants do not close the stomata more quickly than wild-type plants, at least during the initial seven-hour drought period.

Soil water content.

In spite of no significant difference in the number of stomata and in the transpiration rate between wild-type and *fry1-6* plants, *fry1-6* mutant plants still survive longer in drought conditions (**Figure 1**). This might be attributed to a smaller size of *fry1-6*, hence less total transpiration per plant (**Figure 2B**), which leads to slower depletion of water in the pots. If this is the case,

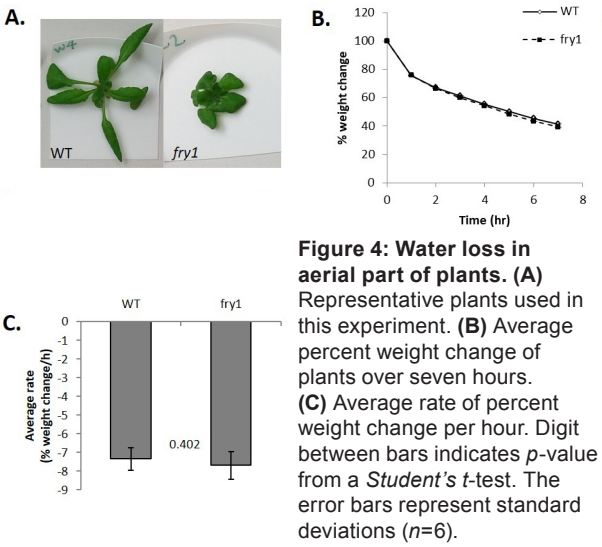

Figure 4: Water loss in aerial part of plants. (A) Representative plants used in this experiment. **(B)** Average percent weight change of plants over seven hours. **(C)** Average rate of percent weight change per hour. Digit between bars indicates *p*-value from a *Student's t*-test. The error bars represent standard deviations (n=6).

fry1-6 plants may have survived longer due to higher soil water content, but not due to a drought-resistant trait of the plant. Therefore, we monitored the soil water content during the course of a drought test. *fry1-6* plants started to show visible signs of dehydration (wilting) in the leaves four days later compared to wild-type plants (day 16 for *fry1-6* vs. day 12 for wild-type; **Figure 5A**). Moreover, at the time when plants started to show such dehydration, the soil water content was significantly lower for the pots with *fry1-6* plants (4.1 g for *fry1-6* vs. 8.8 g for wild-type; $p = 0.0001$; **Figure 5B**). In other words, *fry1-6* plants can tolerate the drought condition until the soil water content reaches down to 4.1 g (9.8 % of average soil dry weight 43.2 g), whereas wild-type plants cannot withstand the drought when the amount of water in the soil is below 8.8 g (20.3 % of average soil dry weight 41.7 g) in the same size of pots. These data suggest that *fry1-6* plants can withstand lower soil water content than wild-type plants, and therefore are more tolerant to droughts.

Discussion

In this study, we aimed to test a new allele of *fry1* mutant (*fry1-6*) for its drought tolerance and understand the causes behind the observed drought resistance. We discovered that the *fry1-6* knock-out allele is also drought resistant, as are other *fry1* alleles carrying base substitution mutations. Despite the clear drought-tolerant phenotype (**Figures 1** and **5**) and the high cellular water content in *fry1-1* and *alx8* (17), the physiological tests we conducted, including assessment of transpiration rates, numbers of stomata, leaf water loss rates, and soil water contents, could not support the hypothesis that the *fry1-6* drought resistance is attributed to reduced transpiration or reduced water usage by mutant plants.

Since the transpiration study we conducted was not reported in the previous study on *fry1-1* and *alx8* (17), these data are new information for the characterization of *fry1* mutant phenotype. The data indicating lower levels of absolute amount of transpiration per *fry1-6* plant (**Figure 2C**) suggests a slower rate of water loss

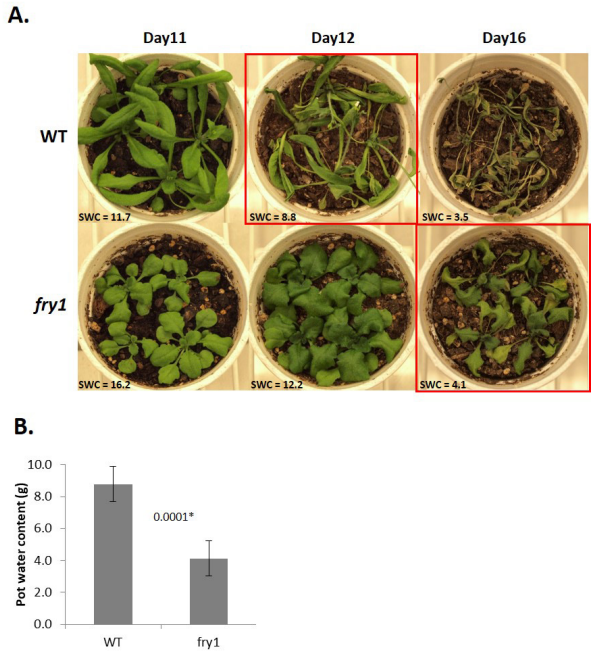

Figure 5: Water loss in whole pots. (A) Representative pots used in this study. Red boxes indicate the pots started to show noticeable signs of dehydration (wilting) in plants. SWC = average soil water content in gram per pot. **(B)** Water content per pot in grams at the time of the onset of visible dehydration (Day 12 for wild-type and Day 16 for *fry1-6* plants). The error bars represent standard deviations (n=5 pots). Digit between bars indicates p-value from a *Student's t*-test.

from the soil in pots containing *fry1-6* plants (**Figure 5**). On the other hand, the similar or slightly higher levels of relative amount of transpiration per unit area (**Figure 2D**) are well supported by the similar numbers of stomata (**Figure 3**) and by the similar rates of leaf water loss (**Figure 4**). This implies that the amount of water that passes through the plant from the root to the air outside of leaves might be quite similar between *fry1-6* and wild-type plants when measured per unit leaf area, which does not support our original hypothesis.

On the other hand, the data also suggests another idea that is not fully tested by our experiments. We found that the transpiration rate per unit area for wild-type plants decreases sharply between 0% PEG and 1% PEG concentrations (**Figure 2D**), however, there is a much smaller decrease in the transpiration rate between 1% and 2% PEG concentrations, and practically no difference in the average transpiration rate in PEG concentrations of 2%, 5%, and 10% (rate stays around 4 mg/h/cm^2). In contrast to wild-type plants, the transpiration rate per unit area for *fry1-6* plants exhibited a slight tendency of consistent decrease as the concentration of PEG increased. This may continue until *fry1-6* plants' transpiration rate per unit area becomes less than wild-type plants. Based on this information, it is plausible that *fry1-6* plants are better able to adapt their transpiration rate under severe drought conditions than wild-type plants. However, due to large variations among the data, the differences between *fry1-6* and wild-type plants are not statistically significant. Also, our leaf water loss test (up to seven hours) did not indicate any difference between *fry1-6* and wild-type plants in losing their water (**Figure 4**). Nevertheless, while the above-mentioned possibility is a pure speculation, this might happen in plants grown on soil in which the extent of dryness becomes extremely severe at the end of a two-week long drought period. If this is true, wild-type plants, faced with the gradual onset of the increasingly severe drought conditions, will be at a survival disadvantage compared to *fry1* plants, since *fry1-6* plants will continue to reduce their transpiration rates to retain water more efficiently whereas wild-type plants will not. In other words, wild-type plants may react better to the onset of less severe drought conditions, but ultimately *fry1-6* plants will be able to adjust better yet than wild-type plants under more severe drought conditions. This may have been the reason for better drought tolerance in soil-grown *fry1-6* plants. Unfortunately, our transpirometer setup was not suitable to simulate soil with a long-term and gradual effect of drought conditions.

Another plausible explanation is that *fry1-6* plants have more osmoprotectant molecules than wild-type plants, allowing them to better retain the water in the cell. Various carbohydrates are known to function as osmoprotectants that help cells adjust the osmotic potential to prevent water loss (23). There are more unidentified sugars found in *fry1-1* and *alx8* plants compared to wild-type plants (17), which may function as osmoprotectants. Likewise, *fry1-6* plants may well have higher levels of osmoprotectants, which can explain why *fry1-6* plants are drought tolerant (**Figures 1** and **5**), as shown in *fry1-1* and *alx8* (17), while exhibiting the same level of transpiration as wild-type plants (**Figure 2D**). Here, we propose that the amount of water that passes through the plants per unit leaf area is not significantly different in *fry1* mutants and in wild-type plants, but the amount of water that is retained inside plants at a given moment is higher in *fry1* mutants due to abundant osmoprotectants. A metabolite profiling experiment in *fry1-6* mutant will address this question, as in the study of *fry1-1* and *alx8* plants (17). On the other hand, we do not rule out the possibility that the transpiration rate in *fry1* mutants can indeed be lower than the one in wild-type plants at a later stage of drought treatment. On-soil transpiration rate tests at a later stage of drought stress will be needed to address this question.

Our study found that *fry1-6* plants did exhibit increased drought tolerance in comparison to wild-type plants. However, we could not pinpoint the exact cause of this trait and instead ruled out several possibilities for the cause of drought tolerance, such as a difference in the number of stomata or a difference in transpiration rates. Further experiments will be needed to discover the exact mechanism of the drought tolerance in *fry1* mutants.

Methods

Plant material and growth.

Wild-type and *fry1-6* mutant plants used in this study

were *Arabidopsis thaliana* ecotype *Columbia*. The *fry1-6* mutant has a T-DNA insertion in the *FIERY1* coding region (15). Seeds were sown on MS medium (24) in a Petri dish and stratified in a refrigerator (4 °C) for three days. Seeds were then germinated and grown at 22 °C in a growth chamber programmed for 24 hours light (cool white fluorescent light with the intensity of 100 μM/sec/m^2). One-week old seedlings were transplanted into soil for further growth under the same environmental condition until used for experiments.

Transpiration rate in varying PEG solutions.

In order to measure the transpiration rate, home-made transpirometers were created by cutting 5 ml pipettes (**Figure 2A**). The bottom segments of pipettes (about 10 cm) were used as the container of a transpirometer. Plants (3.5 weeks old) with soil were removed from the pots and carefully washed off most soil using distilled water. The root part of a single plant was placed into a single transpirometer so that the aerial part was above the transpirometer. The hole at the bottom of the pipette was sealed with Parafilm M (Bemis, Oshkosh, WI). Distilled water was then poured into the transpirometer until about 5 mm from the rim. A thin layer of vegetable cooking oil was placed on top of the water to prevent evaporation from the surface. Drought conditions were simulated by forming hypertonic conditions using various concentrations of PEG (poly[ethylene glycol], MW 8000) solutions (0 % ~ 10 %) instead of pure water. Control transpirometers were set up in the same way but did not contain plants. The transpirometers with plants were placed in a tube rack within a growth chamber using the same environmental conditions as described above. The weight of the whole transpirometer with a plant was measured using a fine balance every 30 minutes for 3 hours. The amount of weight loss reflects the amount of transpiration. Using the data obtained from each plant, a graph was created in Microsoft Excel. The cumulative weight change data were plotted against the time, which showed linear relationship. The transpiration rate (mg/h) was determined through the trend line of the data. The transpiration rate was divided by the plant's surface area (see below) to obtain the transpiration rate per unit area (mg/h/cm^2). Five plants were used for each PEG concentration per genotype (wild-type or *fry1-6*).

Measurement of leaf surface area.

All leaves of the identical plants used in the transpiration experiments were cut off immediately after conclusion of the experiment. Subsequently, the plant leaf blades were unfolded, taped to a white sheet of paper, and photographed. Using ImageJ (22), the leaf surface area was measured and used to calculate the rate of transpiration per unit area.

Number of stomata.

Clear nail polish was applied to the underside of the largest leaf of a plant (3.5 weeks old) and set in a growth chamber to dry. When dried, the nail polish was carefully removed from the leaf and placed on microscope slides. The stomatal imprints were observed using an Olympus BX 41 inverted microscope (Tokyo, Japan) at 200x magnification, and the digital images were taken with a CCD camera attached to the microscope. The number of stomata in each field of view was determined by counting them using the ImageJ program (22).

Leaf water loss test.

The experiment was carried out according to Verslues *et al.* (21). The aerial part of a plant (3.5 weeks old) was cut so that all rosette leaves were attached together. Each prepared plant was placed on a small plastic tray (3 cm x 3 cm) and placed in a growth chamber with the same environmental conditions described above. The weight of each plant was measured using a fine balance every 60 minutes for seven hours and converted to the percentage of the initial fresh weight of the plant. The rate of water loss (% loss per hour) was calculated for each plant using the trend line as described above. Mean and standard deviation of six individual plants per genotype were calculated.

Water Content of Soil.

Seeds were sown and germinated on MS medium (24) as described above. Four one-week-old *fry1* plants were planted in a pot. Five of such pots were used for a total of twenty plants. The same was done with wild-type plants. Plants were grown under the standard condition as mentioned above. After a week, the pots were saturated with water, then left to drip excess water for one hour on a grid shelf. Each pot was weighed afterward (in g). This was recorded as the Day 0 weight measurement, and water was withheld thereafter. Weight measurements in grams were taken every other day until the tenth day, after which measurements were taken every day. After all plants had died, pots were left in an oven at 50 °C overnight to completely dry. The dry pots were then weighed to obtain a dry weight. The dry weight of the pot was subtracted from the previous measured weights of the pots to isolate the weight of water in each pot.

Statistical tests

In this study, all statistical significance between the two genotypes was confirmed through *Student's t*-tests (two-sample unequal variance) by using Microsoft Excel.

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