RESEARCH PAPER

Intrinsically Disordered Proteins Volume 1 e26372; 2013; © 2013 Landes Bioscience

A NAD(P) reductase-like protein is the salicylic acid receptor in the appendix of the Sauromatum guttatum inflorescence

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Keywords: conformation, hydrogel; NAD(P)-reductase like protein; oscillation, salicylic acid, *Sauromatum guttatum*, thermoregulation, volume phase transition

Abbreviations: ACN, acetonitrile; ASA, aspirin, acetylsalicylic acid; CSD, charge state distribution; D-day, the day of inflorescence opening and heat production; 2,6-DHBA, 2,6 dihydroxybenzoic acid; DLS, dynamic light scattering; ESI-MS, electrospray ionization-mass spectrometry; IP, isopropanol; RP-HPLC, reversed phase-high performance liquid chromatography; RL, NAD(P) reductase like protein; SA, salicylic acid; TFA, trifluoroacetic acid

The mode of action of the thermogenic inducers (salicylic acid, aspirin, and 2,6-dihydroxybenzoic acid) in the appendix of the *Sauromatum guttatum* inflorescence is poorly understood. Using ESI-MS and light scattering analysis, we have demonstrated that NAD(P) reductase like protein (RL) is the salicylic acid receptor in the *Sauromatum* appendix. RL was self-assembled in water into a large unit with a hydrodynamic diameter of 800 nm. In the presence of 1 pM salicylic acid, RL exhibited discontinuous and reversible volume phase transitions. The volume phase changed from 800 to 300 nm diameter and vice versa. RL stayed at each volume phase for ~4–5 min with a fast relaxation time between the 2 phases. ESI-MS analysis of RL extracted from appendices treated with salicylic acid, aspirin, and 2,6-DHBA at a micromolar range demonstrated that these compounds are capable of inducing graded conformational changes that are concentration-dependent. A strong correlation between RL conformations and heat-production induced by salicylic acid was also observed. These preliminary findings reveal structural and conformational roles for RL by which plants regulate their temperature and synchronize their time keeping mechanisms.

Introduction

Heat production by the appendix (a 20-cm-long, slender organ) of the *Sauromatum guttatum* inflorescence is well-documented in the literature.[1,2] The generally accepted mechanism of heat-production in the *Sauromatum* appendix is that the alternative oxidase in the mitochondria is the heat-generating enzyme.[3] The alternative oxidase accepts electrons directly from the reduced ubiquinone, and consequently, the proton pumping of the cytochrome pathway is bypassed and, therefore, energy is released as heat.

There are 3 phenolic compounds capable of activating the process that simultaneously leads to heat- and odor-production in a pre-mature *Sauromatum* appendix 24 h after application.[4] These compounds are: SA, ASA, and 2,6-DHBA. SA is the only natural inducer of heat-production that has been identified in the *Sauromatum* appendix, and its mechanism of action in thermogenic plants has never been elucidated.

Salicylic acid is present in many thermogenic as well as non-thermogenic plants, and it exerts its effect on more than one cellular activity in plants. The acid, in addition to triggering the thermogenic respiration in the *Sauromatum* appendix, acts as an endogenous signal in the resistance of many plants to a wide array of pathogens.[5,6] SA in non-thermogenic plants activates a kinase[7] and binds to 2 proteins of 25 kDa[8] and 650 kDa.[9] The acid is also involved in many other plant functions.[10]

In our previous publications, we have described the isolation and purification of a novel 34.1 kDa NAD(P) reductase like protein (RL) from the *Sauromatum* appendix[11] We also have shown that RL is present in 2 global conformations during ESI suggesting that RL conformation was sufficiently maintained for characterization in the gaseous environment of the ESI.[12] An extended conformation (state A) that was only present at pre D-day (prior to the day of heat-production) when SA is undetectable, and a compact conformation (state B) that was present

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Submitted: 07/19/2013; Revised: 08/30/2013; Accepted: 09/03/2013; Published Online: 09/19/2013
http://dx.doi.org/10.4161/idp.26372

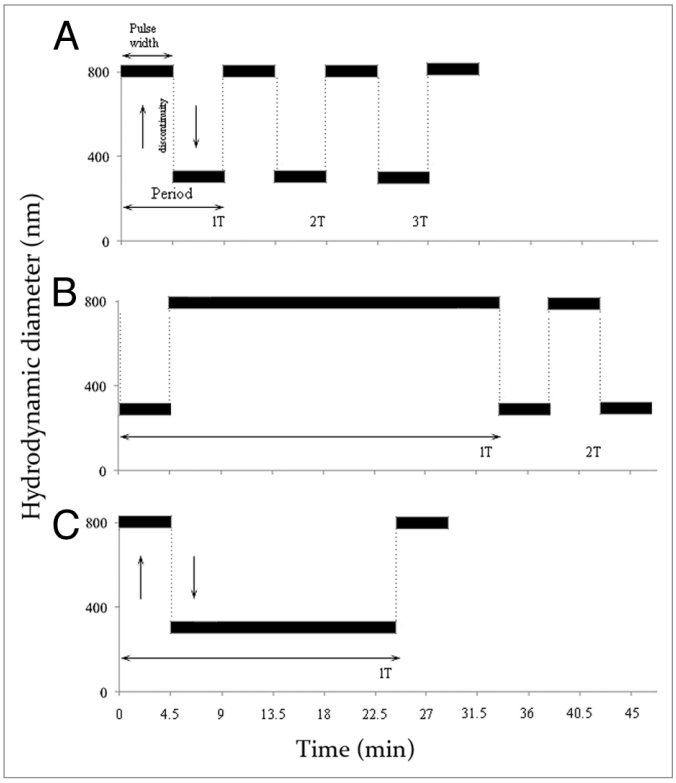

Figure 1. A square wave relaxation oscillation of RL. RL was purified from D-day appendices using RP-HPLC-ESI-MS. Hydrodynamic behavior of 0.1 pM purified RL solubilized in distilled water in the presence of 1 pM SA (**A**), in the presence of 1 pM SA and 10 μM ATP (**B**), and in the presence of 10 μM DTT (**C**). The measurements were carried out using DLS. T, period.

on D-day (the day of heat-production), when SA level is high.[4] Furthermore, a complex self-assembly of RL molecules into large organized structures has been demonstrated implying that RL may be found in the tissue as an oligomer.[12]

The present paper demonstrates that treatment of pre D-day appendices with SA, ASA, and 2,6-DHBA induced reversible conformational changes in RL molecules in a concentration dependent manner. These conformational changes were detected using ESI-MS and DLS.

Purified RL in SA solution behaved as a hydrogel undergoing reversible volume phase transitions from a hydrated to a dehydrated state as determined by DLS. The transitions occurred at a pM concentration range for both RL and SA, and they constituted an ultrasensitive switch-like response[13,14] in which RL switched between 2 discontinuous states. The ultrasensitive response was fast and most interestingly, the transition from one state to the other occurred every 4–5 min.

RL in vivo and in vitro behaved as a bi-stable protein. Bistability may result from different mechanisms, such as multistep phosphorylation,[15] positive feedback loops,[16] and double-negative feedback loops.[17] These types of response can also be reversible.[18,19] Examples of proteins present in 2 different conformations in the cell are the prion protein,[20] the spindle checkpoint protein,[21] the lymphotactin protein,[22] and the cytoskeleton proteins.[23] The presence of a second conformation has a dramatic effect on the function of these proteins. For example, the conformational transition of the prion protein results in a conformational disease in the brain.[20] We conclude that RL binds SA in the appendix tissue, and as SA level rises to a micromolar range, heat is generated.

Results

Induction of a discontinuous volume-phase transition of RL by SA

Purified RL has a great tendency to form large organized aggregates that can be observed under light microscopy. To characterize these aggregates RL was solubilized in distilled water immediately after purification at a final concentration of 0.1 pM and the hydrodynamic diameter of its aggregates was determined in the presence and absence of 1 pM SA using DLS. The low concentration of both SA and RL was used to avoid non-specific interactions between RL molecules and SA. Under this condition, a uniform hydrodynamic diameter of ~800 nm was observed in the absence of SA. Upon addition of SA at a final concentration of 1 pM a discontinuous volume change to a 300 nm diameter occurred. The discontinuous volume change was reversible and it oscillated between 800 and 300 nm diameter with an identical time for each phase, 4–5 min (**Fig. 1A**). This discrete volume discontinuity from 800 to 300 nm diameter was watched for more than 30 min. This effect can only be attributed to the binding of SA to RL molecules. SA is a weak acid and is charged in distilled water but it is unlikely that it changed the pH of the distilled water (pH5 to 6). The relaxation time between the 2 states was fast and could not be determined under our setup parameters.

The reversibility of the volume change resembles a relaxation oscillation with 2 time variables, a slow one when RL was present in either one of the 2 phases, ~4–5 min, and a fast one when RL was switching between the 2 phases. These discontinuous transitions with equal time period may provide a time keeping mechanism in which the phases and relaxation time (discontinuous transition) between phases can be modulated.

To test whether addition of charge would affect the phase transition of RL, ATP which has several negatively charged groups and it is present as ATP^{4-} in water, was added to 0.1 pM RL solution at a final concentration of 10 μM in the presence of 1 pM SA (**Fig. 1B**). Upon addition of ATP, the switching between the phases stopped for about 30 min and RL had a 800 nm diameter after which, switching resumed with the same rhythmic kinetics. It created a temporarily single rectangular wave. The addition of ATP that is negatively charged may temporarily interrupt the crosslinking between RL molecules and allow water molecules to interact with RL molecules. Such a delay in phase transition may allow synchronization with other oscillators with different phase and/or time delay in one single cycle of volume-phase transition.

To determine whether disulfide bonds were involved in the formation of the aggregate, 10 μM DTT or β-mercaptoethanol was added to the RL solution in the absence of SA (**Fig. 1B**). The 800 nm diameter of the aggregate contracted and condensed to ~300 nm diameter in about 4–5 min, and after 20 min in the

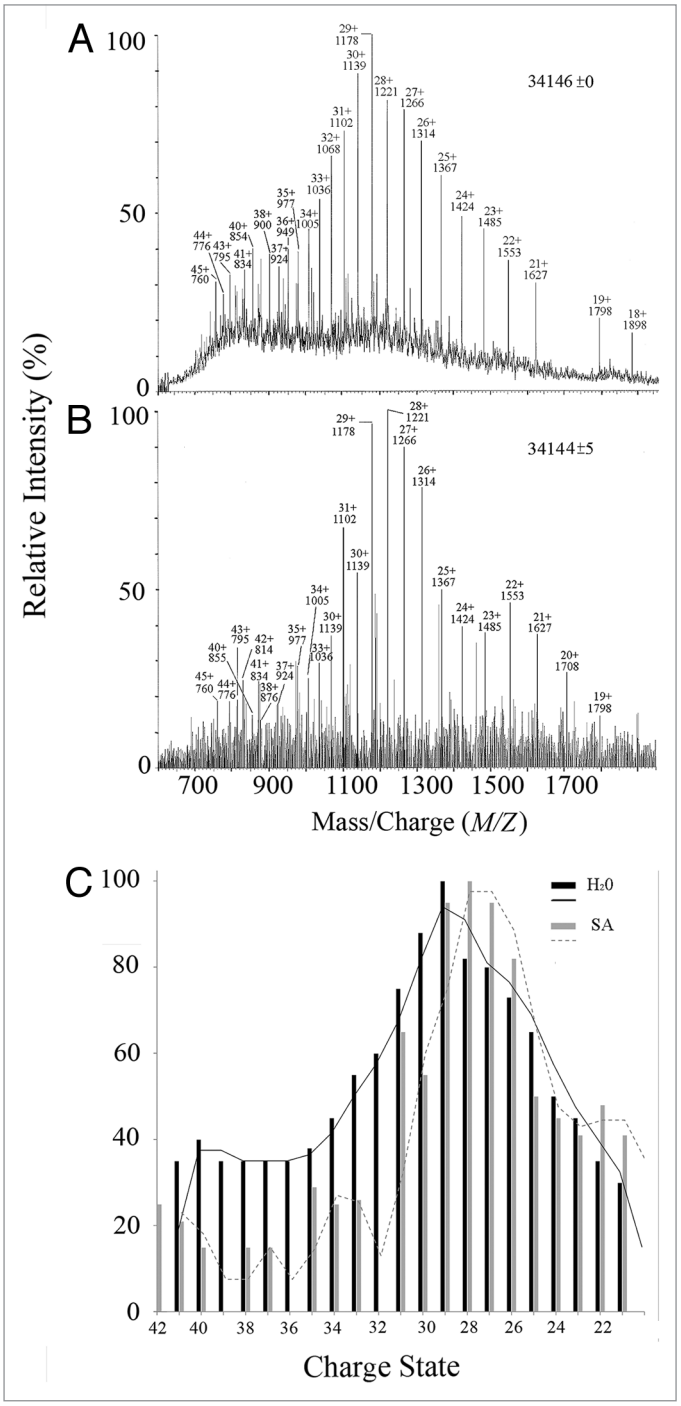

Figure 2. SA effect on RL conformation using ESI-MS. A D-1 appendix was divided into 2 sections. One section was placed in water (**A**) and the other in 10 μM SA (**B**). After 24 h RL was extracted and purified using RP-HPLC/ESI-MS. CSD of RL centered around [$[M + 28H]^{28+}$ for the treated appendix and around $[M + 29H]^{29+}$for the untreated appendix. For an easy comparison the data from **A** and **B** were plotted side-by-side (**C**). Columns in (**C**) denote envelopes of RL ions from **A** and **B**- and trend lines based on moving average of two data points of RL ions were plotted on top of each envelope. Intensities are relative to the 100% maximum peak height in **A** and **B**-.

dehydrated phase the volume went back to 800 nm diameter, and it did not resume a rhythmic kinetic. It suggests that DTT reduced disulfide bonds between cysteine residues required for RL switching. It converted the square wave oscillation to a rectangular wave in which the 2 phases had an unequal period.

This set of experiments suggests that (1) a fixed phase relationship is needed for oscillation; (2) the hydrated and dehydrated phases can be under different control because they react differently to different treatments.

Induction of graded conformations of RL by thermogenic inducers

RL was extracted from appendix sections treated with thermogenic inducers for ~24 h until heat was generated. The data obtained by ESI-MS analysis of purified RL is for the monomeric RL since the protein was in 70% ACN/IP solution containing 0.05% TFA when it was injected into the ESI. Usually, under these conditions oligomirization does not occur. The ESI-MS spectrum of RL from a pre-mature appendix tissue treated with 10 μM SA for 24 h shows the appearance of a CSD centered at $[M + 29H]^{29+}$ and $[M + 28H]^{28+}$, a more compact conformation that previously was called state B (**Fig. 2B**). The RL envelope from a D-1 appendix tissue treated with distilled water (control) was at intermediate state, between state A (a less compact conformation) and B, and the CSD centered at $[M + 29H]^{29+}$(**Fig. 2A**). This appendix section did not produce heat whereas, the treated one produced heat. The CSD of RL shifted to the right of the envelope after application of 10 μM SA solution relative to the untreated D-1 appendix (**Fig. 2C**). It seems that the appendix tissue response to SA treatment resulted in the appearance of a more compact conformation of RL.

ESI-MS analysis of RL extracted from appendix tissue treated with high concentrations of inducers shows that RL underwent conformational changes that resulted in a more expanded conformational than state A (**Fig. 3**). The most profound conformational change was observed with of RL from an appendix tissue treated with 100 μM 2,6-DHBA. It shows the appearance of a CSD centered at $[M + 40H]^{40+}$and $[M + 42H]^{42+}$and the control, untreated one centered at $[M + 36H]^{36+}$, a shift to the left of RL envelope. RL envelope shifted significantly to the left at 200 μM SA. In contrast, application of 200 μM ASA did not shift RL envelope to a more expanded conformation. The hydrated and dehydrated forms of RL molecules detected by DLS may represent the 2 conformations detected by ESI-MS. The hydrated form of RL molecules may represent state A, the expanded conformation, and the dehydrated form of RL molecules may represent state B, the compact conformation.

Figure 4 shows the shifts in the directions of the RL envelope as a result of various treatments with the inducers. SA at 100 μM imprinted a CSD centered at $[M+37H]^{37+}$and at 200 μM at $[M+39H]^{39+}$. It seems that as the concentration of SA increases the shift to the left to a more expanded conformation increases as well. At 100 μM SA the right side of the RL envelope did not change and it may suggest the presence of an additional conformation that is more compact. At 200 μM SA, the CSD of the control disappeared and the shift to the left was more profound. The ESI-MS spectrum of RL treated with 100 μM and 200 μM

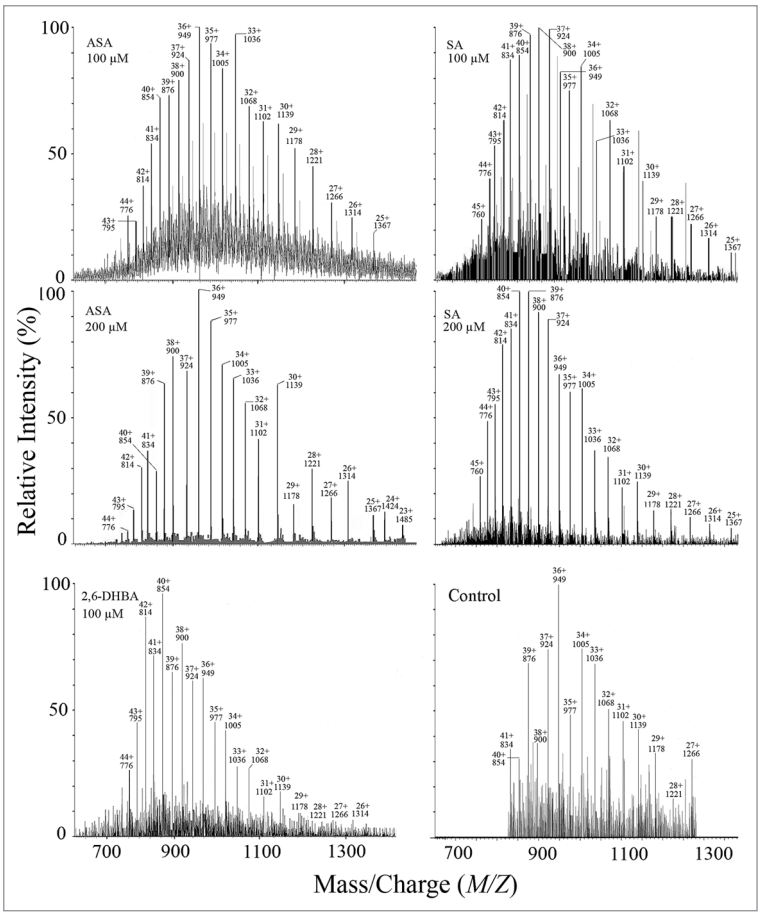

Figure 3. Effect of SA, ASA, and 2,6-DHBA on RL conformation using ESI-MS. Sections of D-2 and D-3 appendices were incubated either in 100 or 200 μM solutions of one of the inducers. After 24 h RL was extracted and purified using RP-HPLC/ESI-MS. CSD of RL centered around $[M + 36H]^{36+}$ for appendices treated with 100 and 200 μM ASA. CSD centered around $[M + 38H]^{38+}$ and $[M + 37H]^{37+}$ for appendices treated with a 100 μM SA solution, and $[M + 40H]^{40+}$and $[M + 39H]^{39+}$ for appendices treated with 200 μM SA solution. CSD centered around $[M + 40H]^{40+}$ for appendices treated with 100 μM 2,6-DHBA solution, and around $[M + 36H]^{36+}$for RL from untreated appendices, control. RL was extracted from 32 g appendix for 100 μM 2,6-DHBA treatment; 35 g appendix for 100 and 200 μM ASA treatments; 39 g for 100 μM and 200 μM SA treatments; 31 g for the control.

ASA shows the appearance of a CSD centered at $[M+36H]^{36+}$as the control but the intensity of the protein ion peaks was higher than the control. This may suggest either a balance distribution of states A and B that results in higher peak intensities on the right and left sides of the RL envelope or, a better protonation of RL molecules after ASA treatment than the control. These results clearly demonstrate that in vivo, at the micromolar range, RL molecules undergo conformational changes that are dependent on both time and inducer concentrations. The result of the graded response in vivo is a significant reduction (in the order of 10^6) in RL sensitivity to the inducers relative to the in vitro response.

SA induces a heat wave

Under the assumption that RL may signal in the cell in a graded manner, heat production was determined at incremental steps of 10 μM. At inducer concentrations higher than 1 μM, thermogenic response was detected using thermocouples (**Fig. 5**). The changes in temperature were slow and the time for reaching a peak was about 6–8 h. The maximum amplitude of the heat waves fluctuated between 0°C to 6°C at different SA concentrations. However, application of 10 μM SA was enough for a conformational change from state A to state B (**Fig. 3**) as well as for a temperature rise (**Fig. 5**). No transition to state B was observed at 100 and 200 μM SA and the appendix tissue stayed at ambient temperature.

The heat waves also shifted backward and forward in time relative to the beginning of the experiment. The appendix sections reached maximum temperature at different times, between 23 to 28 h from the exposure to SA treatment. The temperature profiles changed among appendix sections obtained from 1 inflorescence and among different appendices. Since SA was constantly supplied to the appendix sections, its concentration in the tissue did not fluctuate and therefore it was not the reason for the random outcome of the temperature profiles. One possible explanation is that the decrease in the sensitivity of RL to the inducers in vivo may cause variation in heat-production in which state A is preferable over state B. Temperature fluctuations were observed in more than 50 inflorescences.

Discussion

Square wave oscillation of RL hydrogel

RL occupies large volumes for a 34 kDa protein. The Stokes radii for the folded and unfolded conformations of bovine α-lactalbumin with a molecular mass of 14.2 kDa are ~1.9 and 2.5 nm, respectively.[25] This means that RL is in an oligometric form in water and that RL hydrogel has a high water content. The swelling of a polymer gel is usually determined by the interaction between the oligomer molecules and its affinity for water.[26] Water molecules prevent the collapse of the oligomer network and they create a microenvironment different from the surrounding environment. There are several proteins that can form hydrogel, including NCK (non-catalytic region of tyrosine kinase)/WASP (Wiskott-Aldrich syndrome protein) family proteins and nephrins, which, are involved in actin signaling.[27,28] Artificial proteins, have also been used to form hydrogels.[29,30]

Hydrophobic interactions, hydrogen bonds, and van de Waals forces can be involved in the oligomerization of RL. The water in this kind of gel can be bound, free, or interfacial, and in most cases the interaction with water molecules is reversible. In general, water is an active component in protein conformation[31] and self-assembly proteins.[32]

The thermogenic inducers can replace interactions between RL side-chains and/or backbone with water, and consequently structural reorganization can take place. SA is a weak acid and is negatively charged in water. It interacts with several amino acids residues of human serum albumin suggesting redundant binding sites to proteins with a preference for His, Trp, Lys, or Arg.[33] Non-charged SA forms the most stable complexes with

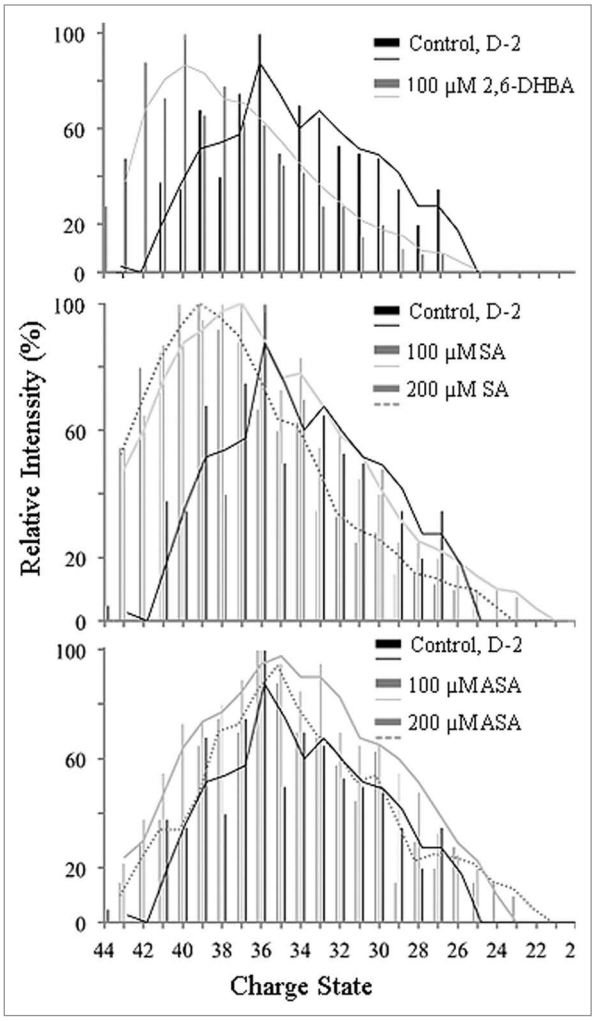

Figure 4. Side-by-side comparison of the effects of inducers on RL conformation. The data from **Figure 2** were plotted side-by-side for an easy comparison of the conformational changes induced by SA, ASA, and 2,6-DHBA. The black columns are the control envelope from **Figure 2** and the gray columns represent an envelope obtained after an inducer application. Trend lines based on moving average of protein ions were plotted on top of each envelope. Intensities are shown relative to the 100% maximum peak for each envelope. The results are noticeably different for each inducer. A strong shift to the left, to a more expanded state is observed with 2,6-DHBA. For SA, the 200 µM shifted the envelope to the left and this shift seems to be similar to the 100 µM 2,6-DHBA. For ASA, the envelope did not shift, and only the intensity of the peaks increased as a result of more surface exposure and protonation of basic amino acids.

polar amino acids residues. Charged SA binds preferably to basic amino acid residues, and at the same times destabilizes non-polar interactions with neutral and hydrophobic amino acids. Binding to His and Trp is stable with either non-charged or charged SA. The protein backbone also affects SA binding. Helical peptides such as poly-Lys and poly-Glu exhibit the highest affinity for SA.

The existence of 2 separate phase and their reversibility can give rise to 2 threshold levels and 2 sudden discontinuous transitions (subcritical Hopf bifurcations), from hydrated phase to dehydrated one and vice versa. The hydrated phase is stable until a threshold is reached, whereby it switches to a more condensed phase in an all-or-none response. The dehydrated phase remains stable until it passes another threshold and only then it become hydrated.

Polymer hydrogels usually undergo a volume phase transition as a response to environmental stimuli such as temperature and small molecules.[34,35] In our case, SA is the stimulus that induces volume phase transition of RL. The volume phase transition of RL requires extremely low concentrations of SA, at a picomolar range. To the best of our knowledge it is the most sensitive response to SA published in the literature.

RL hydrogel as a reservoir

It is unclear whether RL is present in the cell in both monomeric and oligomeric forms. RL can act as a reservoir for the storage and transport of compounds of biological importance such as volatiles that attract pollinators. In human mast cells, the matrix of secretory vesicles can be reversibly condensed and uncondensed in the present of a trigger.[36] Another example is the secretory vesicles in algae belonging to the *Phaeocystis*species.[37] It is possible that RL serves as a matrix for storage in the osmiophilic deposits detected in the *Sauromatum* appendix.[38-40] Its properties may allow accumulation of various compounds including odoriferous volatiles that are released during heat-production. If RL molecules were also temperature sensitive as some other hydrogels, it would add another layer of complexity to its function.

The periodic switching between phases and the uniform sizes of RL in each phase strongly suggests a special organized structure. The oligomerization can proceed in different assembly pathways as we have previously shown.[12] Oligomeric interfaces often have significant electrostatic and geometrical shape that can give rise to specific interactions.[41] For example, a new active site at the interface between subunits can be formed.

RL-A tunable 2-way switch

Monomeric RL undergoes a large-scale conformation transition from one state to another in the *Sauromatum* appendix during development.[12] RL envelope shifted from $[M+37H]^{+37}$ and $[M+39H]^{+39}$ (state A) to $[M+29H]^{+29}$ (state B). Application of 10 µM SA to pre D-day appendices induced a transition from state A in which SA undetectable in the tissue to state B in which SA concentration high in the tissue. The concentration of SA in the appendix tissue is ~1 $\mu g g^{-1}$ fresh wt[4] and it is equivalent to ~7 µM, within the range of this experiment. At higher concentrations of SA and 2,6-DHBA (100 and 200 µM) RL adopted conformations that are even more expanded than state A. It strongly suggests that the conformation of RL can switch between expanded and compact conformations depending on the inducer concentration. These conformations are stable since they are detected in ESI droplets. It also suggests that state A may include several expanded conformations depending on the inducer concentrations.

The capability of RL to adopt several conformations resembles intrinsically unstructured proteins or metamorphic proteins that are capable of adopting more than 1 conformation.[42-45] One characteristic of unstructured protein is the formation of hydrogel.[27]

Ultrasensitive and graded response of RL

The oscillatory, ultrasensitive response of RL arises from its conformational flexibility. If RL could undergo discontinuous

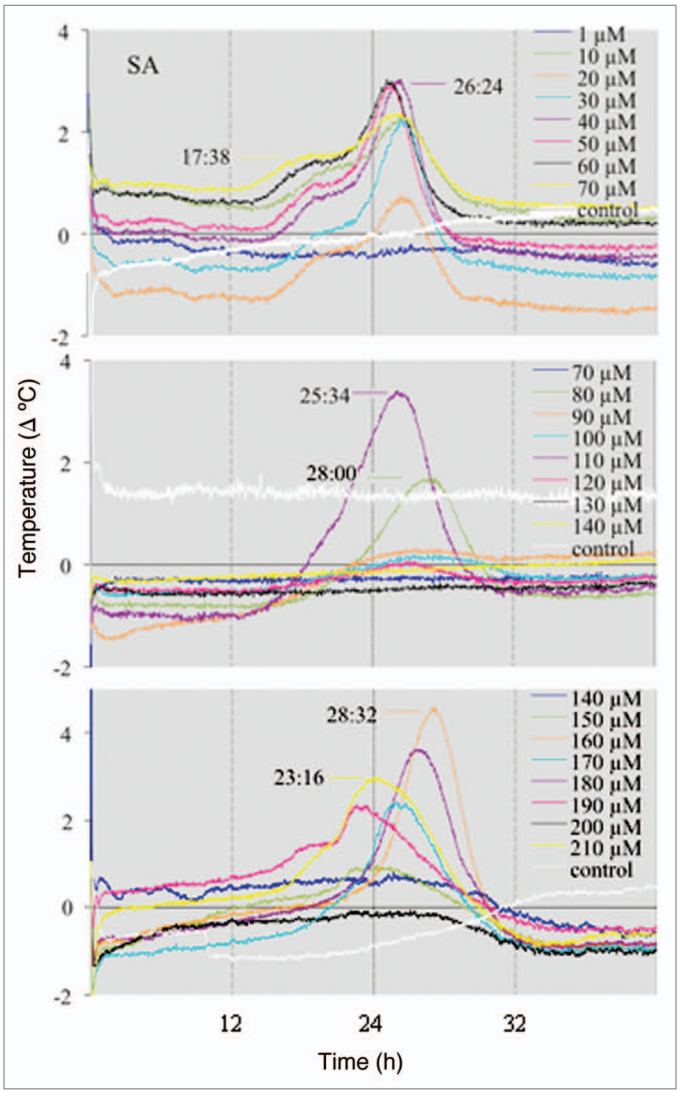

Figure 5. Time courses of heat production induced by SA. Temperature changes in the *Sauromatum* appendix over 48 h at various concentrations of SA were determined. Sections of one appendix were placed in the appropriate concentrations and immediately connected to thermocouples. The effect of SA concentration from 1 to 70 µM was performed with 1 appendix at pre D-day (**A**), and with a second appendix at pre D-day from 70 to 140 µM (**B**), and a third one from 140 to 210 µM. The temperature of the treated sections was subtracted from the control, a section treated with water. Growth chamber temperature was between 20–21°C.

transitions from state A to state B and vice versa every 4–5 min in the appendix tissue, that would imply that its temperature started rising after RL had switch conformation ~259 times (i.e., 19 to 28 h after exposure to SA). This discontinuous transitioncould reduce the sensitivity of RL to SA and consequently heat would not be produced. Only when RL is present in state B for a long time heat can be produced. In order to keep RL in state B, a regulatory negative element(s) that prevents the transition of state B to state A may exist. Furthermore, the temperature rises slowly, over a period of several hours suggesting that the transition from state A to state B is also regulated. If state A is positively regulated (fast transition to state B) and state B is negatively regulated (slow transition to state A), the temperature will rise quickly and the peak will form earlier than 24 h. However, if state A is negatively regulated (slow transition to state B), the temperature will rise slowly and the peak will form after 24 h. It has been shown that ultrasensitive responses are subject to random fluctuations.[46,47] They also generate noise signals that play an important role in different signal pathways.[48]

Temperature sensor oscillator

The strong correlation between the conformation state of RL and heat production led us to conclude that RL plays a major role in heat-production. One putative scenario can be that on D-day the conformation of RL is converted to a more compact one and the stored energy is released as heat. It has been shown that mechanical stress induced in a condensed material can dissipate its energy into heat.[49] The energy stored in the hydrogel may not be substantial to raise the appendix temperature significantly above ambient temperature but it may serve as a signal to transformed chemical energy into heat. The water content of the hydrogel may also affect the thermal conductivity and heat production of the appendix tissue. Water is a good conductor of heat and RL molecules can respond to changes in temperatures. Besides using the heat for the release of odorous compounds, heat can be used as a signal. For example, at high temperatures, MAP kinase cascade is activated and heat shock proteins accumulate.[50]

Another scenario can be that the conformational changes of RL may set the upper and lower temperature limits. State B may set the upper limit and state A the lower limit of the plant temperature. Since RL response is graded and both states are not connected, each temperature limit can be raised or lowered separately and heat may or may not be produced. We have shown that RL in *Arabidopsis* is in state A,[12] a state that does not present during heat-production. In this way RL molecules can keep the cellular temperature within a specific range and maintain energy homeostasis. RL is mainly located in the cytoplasm where it can stimulate glycolysis but it is also present in the mitochondria where it can affect mitochondrial respiration.[11] RL basically adds a level of control and complexity that was undetected so far to the widespread concept of how heat is generated by the mitochondrial alternative oxidase and the uncoupling protein. It is interesting that the timekeeping mechanism is very slow, about 24 h instead of 4–5 min. One can only assume that RL in the appendix tissue is negatively regulated to prevent switching back and forth between the 2 phases.

Clock

The circadian rhythm of the *S. guttatum* appendix seems to be linked to temperature. Heat is produced 25–26 h after application of SA. It deviates from a 24 h cycle and the reason maybe a reset to the correct timekeeping process. The fast transitions are suitable for triggering synchronization of cell energy metabolism and time keeping. Fast modulation of the threshold of relaxation oscillators can lead to synchronization.[51,52] The switch between the 2 phases of RL can act as a biological clock in which switching every 4–5 min can produce a pulse. This clock may operate different than the circadian oscillators described in the literature.[53]

Energy homeostasis

RL is present in the cytoplasm and in the mitochondria as well. Its ability to behave as a switch challenged the conventional assumption that the activity of the alternative oxidase is a simple overflow mechanism. Our data suggest the presence of a system that controls the plant temperature, and therefore, its energy homeostasis. It has also been shown that variants of isoflavone reductase can served as a NADH sensor.[54] It is unknown at the moment if RL molecules change their conformation in the presence of NADH, but it is clear that in the presence of SA, RL induces directly or indirectly a high respiration rate that would result in a lower level of NADH. It is possible that the energy homeostasis of the cell is controlled by changes in RL conformation.

Conclusions and research directions

RL in the presence of SA is a bi-stable protein that behaves as a 2-way switch that can also be converted to a graded switch. These properties are associated with heat-production and the release of odoriferous volatiles. These properties may allow RL to be a signal and to function as a clock, as well as a storage site for biological important compounds. The sensitivity of RL to SA and its properties make it a perfect candidate for being the receptor of SA in thermogenic and non-thermogenic plants. Many more experiments are needed to in order to understand the complex behavior of RL. We hope that these preliminary results will stimulate the research on thermoregulation in plants.

Methods and Materials

Plant material

Corms of *Sauromatum guttatum* were kept at 4°C and the inflorescences were allowed to develop under normal day/night cycle (15L/9D) at room temperature with a photon flux density of 210 μmolm^{-2}sec^{-1}. The developmental stage of the appendix was determined retroactively with respect to the day of inflorescence opening and heat-production (D-day).

Dynamic light scattering

All reagents were filtered through a 0.22 μM filter prewashed with 0.1 N HCl. RL was solubilized in distilled water to a final concentration of 0.1 pM. The scattering light measurements were performed with an argon-ion (λ = 488 nm) laser spectrometer (Brookhaven Instruments) at a 45° scattering angle using 12 ml quartz cuvettes with a 1.5 mm path length. The autocorrelation function of the fluctuation intensity of the scattered light was processed by a Brookhaven BI-9000AT autocorrelator. Particle size distribution was calculated using the CONTIN method.[24]

ESI-MS

A Micromass Quattro II tandem quadrupole MS fitted with an ESI source (Waters, Inc.) via a variable splitter allowing manual fraction collection was used in this study.[11] The instrument was operated in a positive ESI mode at a probe tip voltage of 3.6 kV and a cone voltage of 45 V. The source and nebulizer temperatures were maintained at 150 and 400°C, respectively, using N_2 as both nebulizing and bath gas. The instrument was calibrated using water clusters over the range of 100–2400 Da. Data acquisition was performed from *m/z* 500 to 2000 in the continuum-scanning mode at 450 Da sec^{-1}. Instrument tuning, mass calibration, data acquisition, processing and display were accomplished using MassLynx™ 3.4 and MaxEnt™ software (Waters Inc.). This procedure provided a protein molecular mass with an accuracy of ± 0.1%. The low mass errors reported on individual spectra indicate the precision of the mass determination and therefore its reproducibility. The performance of the ESI-MS system has been verified by the identification of carbonic anhydrase (29,030 Da, Bovine erythrocytes, Sigma-Aldrich Corp.) as a standard.

Induction of a thermogenic response

The *Sauromatum* appendix was cut transversely into equal length sections and immediately placed in an appropriate solution for 24 h for RL purification and ESI-MS analysis and for 48 h for temperature measurements. Sections were placed in aqueous solutions containing different concentrations of thermogenic inducers; SA (Fisher Scientific), ASA (Sigma-Aldrich), 2,6-DHBA (Spectrum), or in distilled water as a control. The inducer solution was prepared as follows: a known amount of inducer was dissolved in 100 μl ethanol and diluted with distilled water to form a 1 mM stock solution that was further diluted with distilled water to an appropriate concentration. The appendix sections in the inducer solutions were kept in an environmental chamber at 20-21°C.

Under these conditions the pK_a of SA which, is a weak acid, is 3.0 at 25°C. Its second dissociable group, the hydroxyl group in position 2, has a pK_a of ~13. The pK_a of ASA is 3.5, and of 2,6-DHBA is 1.3. Therefore SA, ASA, and 2,6-DHBA were charged in distilled water with different ratio between non-charged and charged states. The ratio between non-charged and charged for SA is ~1:100.

Temperature measurements

Precision thermocouples (copper/constantan, Omega) were inserted into appendix sections, and a data logger (Omega) collected the temperature data over a 48 h period at 2 min intervals. The effect of a treatment on heat-production was calculated by subtraction a particular temperature of a water treated section (control) from that of a treated section, and the result was a temperature change triggered by SA at a certain concentration. Temperature measurements were performed under constant light at 20–21°C.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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