Regulation of STY kinases by a metabolite binding ACT domain

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**ABSTRACT**

Protein import of nuclear-encoded proteins into plant chloroplasts is a highly regulated process, requiring fine-tuning mechanisms especially during chloroplast differentiation. One way of altering the import efficiency is achieved by phosphorylation of chloroplast transit peptides in the cytosol. Recently, we have investigated the role of three serine/threonine/tyrosine (STY) kinases, STY8, STY17, and STY46, in precursor phosphorylation. These three kinases share a high degree of similarity and harbor a conserved ACT domain upstream of the kinase domain. The ACT domain is a widely distributed structural motif, known to be important for the allosteric regulation of many enzymes. In this work, using biochemical and biophysical techniques both in vitro and in planta, including kinase assays, microscale thermophoresis, size-exclusion chromatography, as well as site-directed mutagenesis approaches, we show that the ACT domain regulates autophosphorylation and substrate phosphorylation of the STY kinases. We found that isoleucine and S-adenosylmethionine bind to the ACT domain, negatively influencing its autophosphorylation ability. Moreover, we investigated the role of the ACT domain in planta and confirmed its involvement in chloroplast differentiation in vivo. Our results provide detailed insights into the regulation of enzyme activity by ACT domains and establish that it has a role in binding amino acid ligands during chloroplast biogenesis.

As a consequence of endosymbiosis, most chloroplast genes were transferred to the nucleus and the resulting proteins were equipped with N-terminal cleavable transit peptides. A specific subset of these precursor proteins associate with cytosolic compounds, amongst them 14-3-3 proteins for efficient relocation from the cytosol to chloroplast translocon prior to the import process (1-4). Several of these precursor proteins are phosphorylated within the transit peptides at specific serine or threonine residues leading to their association with 14-3-3 proteins. Albeit formation of the precursor-14-3-3 complex enhances import rates, dephosphorylation is required prior to the translocation to ensure efficient import (5,6). Regulation by phosphorylation was shown to be especially important in cotyledons and during chloroplast differentiation (7). Precursor phosphorylation is executed by the cytosolic serine/threonine/tyrosine (STY) kinases in Arabidopsis, i.e. STY8, STY17 and STY46. Mutant lines with deletion of STY8 and STY46 accompanied by knockdown of STY17 causes inefficient chloroplast differentiation, delayed accumulation of chlorophyll, hampered photosynthetic capacity and significant
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reduction of nucleus-encoded chloroplast proteins (7).

A unique feature of these kinases is that they harbor a predicted, but uncharacterized ACT domain located upstream of their kinase domains (7). The ACT domain has been found in several enzymes and thus the name was derived from the first letters of three of the proteins in which it was first identified, i.e. aspartate kinase-chorismate mutase-tyrA (prephenate dehydrogenase)(8). In the meantime, ACT domains have been identified in proteins with a plethora of functions such as control of metabolism, solute transport, and signal transduction (9). The ACT domain ranges from 60 to 80 amino acids and the structural motif comprises four β strands and two α helices organized in a βαβαβαβ fold (10,11). Since the primary sequences are very variable, their identification often requires a combined sequence/structure and also functional analysis (11). Interestingly, ACT domains can vary in number and often mediate oligomerization. The ACT domain has been shown to bind to multiple ligands and generally seems to play a role in allosterically regulating the enzymatic activity of proteins. The ligand types range from amino acids to enzyme cofactors (9,10). In some cases, enzyme activity can be inhibited upon ligand binding, as has been observed in the E. coli D-3-phosphoglycerate dehydrogenase, where binding to its ligand (serine) causes disruption of the substrate binding domain (12). In contrast, phenylalanine stabilizes a hydroxylase upon substrate binding in the ACT domain (13). Interestingly, it has been shown that lysine and the cofactor S-adenosylmethionine (SAM) synergistically inhibit Aspartate Kinase 1 from Arabidopsis via binding to different regions within the ACT domain (14,15).

The STY8, STY17 and STY46 kinases are particularly interesting as they are the only protein phosphorylating enzymes known to contain an ACT domain. Previously we could show that deletion of the ACT domain of STY8 causes increase of autophosphorylation as well as precursor phosphorylation yield in vitro (7). In this study, we examined the role of the ACT domain of the STY kinases using biochemical and biophysical techniques in vitro as well as in planta. We identified isoleucine (Ile) as well as SAM as negative regulators acting on kinase autophosphorylation. Thus, this study presents new insights into the regulation of enzyme activity by ACT domains and elucidates its role with respect to ligand binding during chloroplast biogenesis.

RESULTS

The ACT domain regulates STY kinase activity and substrate binding

As a first step, all three STY kinases, STY8, STY17 and STY46 as well as the corresponding ACT domain deletions STY8ΔACT, STY17ΔACT and STY46ΔACT were expressed in E.coli and purified via affinity chromatography. Autophosphorylation of the six recombinant kinases was investigated using radiolabeled [γ-32P]-ATP. All ACT deletion mutants displayed hyper-autophosphorylation to a similar extent compared to their WT variants (Figure 1A). To analyze substrate phosphorylation, the small subunit of the chloroplast ribulose-1,5-bisphosphatase (pSSU), which is known to be phosphorylated within the transit peptide, was used as a model substrate (1,16). pSSU was expressed and purified as a His-tag fusion protein from E. coli. Phosphorylation of pSSU was also found to be enhanced by all three kinases lacking the ACT domain (Figure 1A). As a negative control we used STY8 T439A, STY17 T445A, STY46 T443A - mutants that exhibits loss of autophosphorylation activity (Figure 1A). Quantification of three independent kinase assays was performed and is shown in Figure 1A.

Next, we set out to investigate the binding affinities of STY8 to its substrate using microscale thermophoresis (MST). For the MST measurements, pSSU was labeled with a fluorescent dye and interaction was analyzed applying increasing amounts of recombinant STY8 or STY8ΔACT. Indeed, strong binding of STY8 to pSSU occurred with a $K_D$ of 8.08 ± 1.4 nM. Intriguingly, the binding affinity was even enhanced with STY8ΔACT to a $K_D$ of 4.20 ± 2.5 nM (Figure 1B). To investigate whether the autophosphorylation state of the kinase directly affects binding affinity to pSSU we also used STY8 T439A. In fact, we obtained the weakest binding affinities of STY8 T439A with pSSU ($K_D = 708 ± 6.5$ nM) (Figure 1B). These data suggest an important role of the ACT domain not only in the regulation of
autophosphorylation, but also indicate that autophosphorylation triggers the formation of a kinase-precursor complex.

**STY8 and as well as the ACT domain form oligomers in vitro**

Several ACT domain-containing proteins have been isolated as large multimeric proteins with ACT domains mediating the formation of dimers, trimers or even tetramers (10). To elucidate the oligomeric state of the recombinant STY8 protein we employed size-exclusion chromatography (SEC). Our results showed that the majority of STY8, which has a monomeric size of 63 kDa, elutes at 217 kDa, suggesting the formation of a trimer or a tetramer (Figure 2A). To investigate the role of the ACT domain in the oligomerization state of STY8, we purified and analyzed only the ACT domain, which has a calculated size of 7.5 kDa. Our results indicate that the purified ACT domain of STY8 likewise elutes as a trimer or a tetramer, due to the apparent size of 35 kDa, thus indicating that oligomerization may be mediated by the ACT domain (Figure 2B). To investigate the oligomerization status further, we applied a non-denaturing Native-PAGE (Figure 2C). The results show a band at approx. 230 kDa, also indicating trimer or tetramer formation.

**STY8 is negatively regulated by Ile and SAM via its ACT domain**

Since amino acids are common ligands of ACT domains, we conducted radioactive kinase assays with STY8 and 20 amino acids at a final concentration of 5 mM. Quantifying the kinase activity via its autophosphorylation levels, we found that Isoleucine (Ile) had a strong negative effect on kinase autophosphorylation (Figure 3A). When applying increasing amounts from 0-5 mM Ile to a STY8 autophosphorylation assay, we observed a decrease in autophosphorylation with increasing amounts of Ile (Figure 3B, upper panel). However, using STY8ΔACT, we observed loss of Ile sensitivity, suggesting that Ile binding indeed occurs within the ACT domain (Figure 3B, lower panel). To analyse the binding of STY8 and Ile on a quantitative level we employed MST. STY8 and STY8ΔACT were labeled with a fluorescent dye, and increasing amounts of Ile were used as analytes. Indeed, we observed a binding event using WT STY8 (K_D = 311 ± 16 μM). However, no binding curve could be fitted using STY8ΔACT, again supporting the idea of the ACT domain as Ile binding site (Figure 3C).

It has been previously reported that, along with amino acids, ACT domains also interact with various metabolites derived from amino acids. Interestingly, the ACT domain of the Arabidopsis aspartate kinase binds to lysine as well as to SAM. Therefore, we analysed the effect of SAM on STY8 and discovered that the metabolite down-regulates STY8 autophosphorylation (Figure 3D). To examine whether SAM affects the kinase activity also via the ACT domain, we incubated increasing amounts of SAM with STY8 and STY8ΔACT and compared autophosphorylation activity (Figure 3D). Indeed, SAM reduces autophosphorylation in a similar manner to Ile and again the activity of STY8ΔACT showed SAM-resistance, implying that SAM also binds to STY8 via the ACT domain. To quantify the binding event we conducted MST analysis with SAM and STY8, or STY8ΔACT. A binding affinity could again be calculated for STY8 (K_D = 774.7 ± 39 μM), but no curve could be fitted for STY8ΔACT (Figure 3E). To verify further that the metabolite binding is conferred by the ACT domain, we used the recombinant ACT domain to perform MST analysis with Ile and SAM. Interestingly, the K_D values were enhanced to 81.48 ± 34 μM for Ile and to 47.28 ± 25 nM for SAM (Figure 3F). This result unequivocally confirms that both metabolites are bound by the ACT domain. The differences observed in binding affinities using the full length proteins versus the isolated ACT domain might indicate that steric hindrances occur in measurements with the full-length proteins thus leading to lower accessibility for the metabolites. Nevertheless, SAM still seems to bind to STY8 with a higher affinity as compared to Ile. To confirm specificity of the binding event, we included methionine as a negative control. No binding event could be observed for these measurements (Figure 3F).

Since we observed that the extent of autophosphorylation influences affinity to the precursor pSSU, we were wondering whether a reduction in autophosphorylation by adding SAM or Ile has a negative effect on precursor binding. Consequently, we performed MST analysis with STY8 and pSSU in the presence of Ile and/or SAM. In this case, pSSU was
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fluorescently labeled and the final concentration of the labeled pSSU and SAM and/or Ile were kept constant at 1 mM. STY8 was titrated against pSSU and the thermophoretic mobility was monitored (Figure 3G). The addition of SAM or Ile had an equal negative effect on the binding of STY8 to pSSU, in both cases the KD was reduced approximately 4-fold. Interestingly, a 10-fold reduction in the binding affinity was observed when SAM and Ile were applied simultaneously (Figure 3G). To investigate if STY17 and STY46 were likewise affected by Ile and SAM we performed autophosphorylation assays in the presence and absence of 5 mM Ile and 5 mM SAM with all three WT kinases, as well as kinases lacking the ACT domain. All three kinases behaved in a similar manner and quantification of the results show that addition of Ile or SAM resulted in a downregulation of autophosphorylation of approximately 50% in all three kinases, while deletion of the ACT domain results in metabolite insensitivity (Figure 3H).

Mutation of a conserved glycine in the ACT domain causes loss of Ile sensitivity

Ligand binding in ACT domains is usually observed at the interfaces between the domains and there appears to be a correlation with the occurrence of a specific glycine residue located in the loop between the first β-strand and the first α-helix of ACT domains (10). For example, a glycine to aspartate mutation of the ACT domain-containing Aspartokinase in *E. coli* revealed a significant loss of amino acid sensitivity (17). To investigate the presence of such a residue in the STY kinases, we performed a structure prediction with the ACT domain of STY8 using the iterative threading assembly refinement (I-TASSER) server. Indeed, we could identify a glycine residue at position 197 in the respective loop area in STY8, which also proved to be conserved in STY17 and STY46, as well as in homologues in other plants species (7, Figure S8). The predicted structure, along with an expected scheme of the ACT domain fold, is shown in Figure 4A. The α-helices are depicted in pink, beta sheets in blue and the conserved glycine is denoted in green, respectively.

To study the role of the glycine residue in question, we substituted the amino acid by aspartate (STY8 G197D) and compared its Ile sensitivity to WT STY8, monitoring autophosphorylation with increasing Ile concentrations (Figure 4B). Intriguingly, we observed Ile insensitivity of the STY8 G197D mutant, similar to STY8ΔACT, indicating that the exchanged glycine is indeed involved in Ile binding. To investigate further whether the STY8 G197D mutant is also insensitive to SAM, a kinase assay was performed with STY8 G197D in the presence of increasing concentrations of SAM. Strikingly, in contrast to Ile, we observed SAM sensitivity in both WT and the STY8 G197D mutant. STY8ΔACT was again used as a negative control, showing that autophosphorylation was not affected by SAM in this mutant (Figure 4C). To verify these results, we performed MST with STY8, STY8 G197D and STY8ΔACT to analyze Ile and SAM binding. Indeed, the binding affinity of Ile to STY8 G197D was reduced to almost 1 mM, whereas the binding affinity to SAM was not affected in STY8 G197D (Figure 4D). By and large, our data indicate that the conserved glycine located in the loop region between the first β-strand and the first α-helix beta sheet is only responsible for Ile binding. Albeit SAM likewise binds to the ACT domain, the binding site must be different. This also explains the additive negative effect observed on STY8-pSSU upon the addition of SAM and Ile (Figure 3G).

Impact of deletion of the ACT domain in planta

To study the role of the ACT domain in planta, we used the previously generated sty8 sty46 double mutant, which shows a reduced growth as well as slowed greening, most probably due to an inefficient import of precursors (7). Since we could show in the respective study that the growth phenotype of the *sty8* *sty46* mutant could be fully rescued by overexpression of *STY46*, we complemented the line with *STY46* as well as *STY46ΔACT* under control of the 35S CMV promoter (*sty8* *sty46*35S::*STY46* and *sty8* *sty46*35S::*STY46ΔACT*). Expression of *STY8* and *STY46* in the generated lines was confirmed using reverse transcription (RT)-PCR. Neither STY8 nor STY46 were detected in *sty8* *sty46*, but *STY46* was again expressed in *sty46*35S::*STY46* and *sty8* *sty46*35S::*STY46ΔACT* (Figure 5A). Since *sty8* *sty46*35S::*STY46* and *sty8* *sty46*35S::*STY46ΔACT* were compared in the following phenotypic studies, quantitative RT-PCR was performed to show that both *STY46* variants were expressed to a similar degree (Figure 5B).
To investigate whether hyper-phosphorylation of the kinase and its substrates could also be observed with sty8 sty46/35S::STY46ΔACT plant extracts, we performed kinase assays using soluble protein extracts of WT plants, sty8 sty46 double mutants, sty8 sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT complementation plants to study the phosphorylation potential of recombinant pSSU. Precursor phosphorylation was reduced in the sty8 sty46 double mutant compared to WT, as expected (Figure 5C). Residual phosphorylation in sty8 sty46 is most likely due to the redundant function of STY17. However, the precursor phosphorylation yield was approx. 2.5 fold higher in sty8 sty46/35S::STY46ΔACT in comparison to WT as well as to 35S::STY46, as shown by quantification of three independent experiments (Figure 5C). We therefore conclude that the STY46ΔACT functions in planta in a comparable manner to the recombinant purified protein.

Since we previously observed delayed greening in sty8 sty46, we were interested to see if deletion of the ACT domain has an effect during etioplast to chloroplast transition. WT, sty8 sty46, sty8 sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT seedlings were grown in darkness for 6 d and subsequently transferred into light for 4 h to investigate differences during chloroplast differentiation. Greening of the cotyledons was monitored by appearance and chlorophyll accumulation was determined (Figure 5D and E, control). As expected, the greening process was significantly delayed in sty8 sty46. Interestingly, this phenotype could only partially be rescued in sty8 sty46/35S::STY46ΔACT, as could be observed by a yellowish appearance as well as a slight reduction in chlorophyll accumulation as compared to WT. sty8 sty46/35S::STY46 plants in contrast, behaved like WT.

Further, we analyzed the phenotype of the generated plants under long day growth conditions for 3 weeks (Figure 5F). Surprisingly, where sty8 sty46 mutants were smaller as compared to the WT, no change in the leaf area was observed between the WT, sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT.

To investigate the inhibitory effect of SAM and Ile on the kinase autophosphorylation in the greening process, WT, sty8 sty46, sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT lines were analyzed on MS plates supplemented with 8 μM SAM and 100 μM Ile (18,19) (Figure 5D). Interestingly, the WT and sty8 sty46/35S::STY46 both showed reduced chlorophyll accumulation as compared to the untreated control, indicating an inhibitory effect of excess SAM and Ile on chloroplast differentiation. However, sty8 sty46/35S::STY46ΔACT plants showed no reaction to SAM and Ile treatment, when compared to the untreated plants (Figure E). We therefore propose that the observed negative effect caused by SAM and Ile treatment in the WT is indeed due to kinase inhibition via the ACT domain. The sty8 sty46 mutant also showed a reduction in chlorophyll accumulation upon SAM and Ile treatment, possibly due to STY17 inhibition.

DISCUSSION

Over the years, ACT domains have gained increased recognition as allosteric regulators of various enzymes. Noteworthy, in this study the regulation of a protein kinase by an ACT domain was investigated for the first time. The ACT domain has been suggested to function as an evolutionary conserved module to mediate various allosteric responses, an idea that is supported by our findings (8,11). Since ACT domains are not very highly conserved on a primary sequence level, they proved to be difficult to identify and classify by simple PSI_BLAST searches. Although structural predictions allow a more precise annotation, a functional characterization including their binding of small molecules as well as their regulatory effect on other catalytic domains was suggested in addition to in silico sequence and structural analysis (11). Indeed, we could provide evidence that the ACT domain in the STY kinases meet these criteria, since it binds to typical small molecules, which results in a direct effect on the kinase activity. In addition, we could show that the ACT domain mediates oligomerization of STY8, yet another typical feature of the respective domain. We therefore conclude that the investigated domain represents a bona fide member of the ACT domain family.

SAM and Ile were identified as binding partners of the ACT domain in this study and were
shown to reduce kinase activity as well as affinity to the substrate pSSU. Interestingly, the precursor of SAM, methionine, as well as Ile are synthesized in the chloroplast. This may indicate a feedback regulation of the requirements of the chloroplast and the cytosolic precursor phosphorylating kinases. The exact physiological events and signals, however, remain to be determined. Moreover, we could show that Ile requires a conserved glycine in the first loop region of the ACT domain. This is in contrast to SAM, which apparently utilizes a second binding site within the ACT domain. We hypothesize that binding of SAM and Ile leads to conformational changes rendering an inactive structural state of the kinase. Such allosteric regulations have been observed previously, for example in the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAH7PS), which catalyzes the first step of the shikimate pathway. Upon inhibition by tyrosine, DAH7PS adopts a closed inactive conformation. Albeit several structures of ACT domains have been obtained, the comparison between structures with and without ligand that would help to unravel conformational switches is often lacking.

The scheme in Figure 6 summarizes the proposed regulatory role of the ACT domain in kinase activity by autophosphorylation and its consequential role in chloroplast development. We assume that in the WT kinase, modest autophosphorylation takes place, which is in turn reflected in a modest phosphorylation of precursors. As we have observed previously that greening of young seedlings is delayed in STY kinase mutants, we suppose that phosphorylation of precursors positively influences import efficiency, possibly by stabilizing the precursor and/or enhancing its affinity to the chloroplast translocon. Nevertheless, we have also observed that precursor dephosphorylation prior to the translocation is vital to ensure efficient import. In the present study, we could show that deletion of the ACT domain results in hyperphosphorylation as well as slower greening in planta when compared to WT. According to our previous observations, we argue that the enhanced phosphorylation cannot be compensated by an equivalent activity of the responsible phosphatase – the nature of which is still elusive. In turn, hyperphosphorylation caused by lack of the ACT domain leads to slower chloroplast to etioplast transition (Figure 6). In line with this we have previously shown, using precursor phosphomimicking mutants, that constant phosphorylation in the transit peptide likewise results in hampered import and chloroplast defects (5). Apparently, enhanced transit peptide phosphorylation is especially important in cotyledons, since mature sty8 sty46/35S::STY46ΔACT plants are undistinguishable from WT. This observation also matches our previous results, where we could show that expression of a phosphomimicking variant of the essential PSII assembly factor HCF136 only partially rescued the seedling lethal hcf136 phenotype. Intriguingly, the reduced import of HCF136 had a significant effect in cotyledons, whereas mature leaves developed similarly to WT (6). Further, application of SAM and Ile during the greening experiment reduced the etioplast to chloroplast transition in the WT and consequently in sty46/35S::STY46 (Figure 6). sty46/35S::STY46ΔACT, however, was not affected by SAM and Ile as compared to untreated plants, demonstrating that SAM and Ile not only affect kinase activity in vitro, but also impact chloroplast development, likely via regulation of the STY kinases. We hypothesize that SAM and Ile binding both lead to a conformational change in the STY kinase, resulting in its reduced activity.

EXPERIMENTAL PROCEDURES

**Plant materials and growth conditions**

*Arabidopsis thaliana* WT Columbia ecotype (Col-0) and the mutants were grown either on soil or on half-strength Murashige and Skoog (MS) medium supplied with 1% sucrose under controlled conditions in a growth chamber. For soil based phenotyping analysis, plants were grown on long-day conditions (16 h/8 h of light/dark, 22°C, 120 μE m⁻² s⁻¹). For plate-based analysis, dry seeds were surface sterilized. Seeds were vernalized at 4°C for 2 d. Mutant plants, SALK 072890 (sty8) and SALK 116340 (sty46) lines were described previously (7). For the generation of complemented sty8 sty46 mutants the WT *STY46* cDNA as well as the mutated cDNAs (*STY46ΔACT*) were cloned into the binary vector pB7FWG2, with a stop codon (Plant Systems Biology using the Gateway system (Life Technologies). The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and the
Homologous sty8 sty46 mutant plants were transformed by floral dip (20).

**Quantitative RT-PCR analysis of transcripts**

Total RNA was isolated from several leaves using the Plant RNeasy extraction kit (Qiagen). cDNA was synthesized from 1 μg of RNA (DNase treated) using iScript™ cDNA Synthesis Kit (BioRad). All reactions were done in triplicates on three biological replicates. The relative abundance of all transcripts amplified was normalized to RCE1 (At4g36800). The RCE1 gene was used as an internal reference in other studies (21,22). For quantitative RT-PCR, the SYBR Green real-time PCR master mix (Roche) was used, and the reaction was performed in a Bio-Rad CFX96 real-time PCR detection system. Forty-five cycles were performed as follows: 1 s at 95°C, 7 s at 49°C, 19 s at 72°C, and 5 s at 79°C. The following oligonucleotides were used: STY8QRT 5'- CATCAGTTCCATCCGTAGGTA-3', STY46QRT 5'- AGGTTTAAAAGATGCATTGA-3', STY17QRT 5'- AGGTGCCAGAACGCATGTTCC-3'. Point mutations leading to single amino acid substitutions were introduced as described (23) with the following oligonucleotides: STY8-G197D_fr 5'- TTGGTGAAGTTGATCAGTCAGTAATGATCGAATA GAG-3'. Cloning of the dead kinases, as well as the overexpression clone for pSSU are described elsewhere (1,7). All constructs were expressed in E. coli BL21-CodonPlus (DE3)-RIPL cells. For protein purification, cells were resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), lysed using a French press, and centrifuged for 30 min at 20000 x g. After centrifugation, the supernatant was incubated with Ni-Sepharose fast flow (GE Healthcare) for 1 h at 4°C. The Sepharose was washed twice with washing buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 10 mM imidazole) and recombinant proteins were eluted by increasing the imidazol concentration to 500 mM.

**In vitro kinase assays**

Recombinant kinase or stroma extract was incubated with recombinant substrate in the presence of 3 μCi of [γ-32P]-ATP and 2.5 μM ATP in a total volume of 25 μL of kinase buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, and 0.5 mM MnCl2). The reaction was carried out for 15 min at 23°C and stopped by adding 5 μL of SDS sample buffer. The proteins were separated on a 12% SDS-polyacrylamide gel followed by autoradiography. Gels were exposed overnight.

**MST analysis**

MST was performed with purified recombinant protein and different ligands. The proteins were diluted in MST buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.05% Tween-20). Labeling of proteins was performed using the Monolith His-Tag Labeling Kit RED-Tris-NTA (NanoTemper, Munich). Tris-NTA dyes were diluted in PBST buffer (137 mM NaCl, 2.5 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4, 0.05% Tween-20) to a final concentration of 100 nM. Increasing concentrations of non labeled protein (0.15 nM - 5 μM) or metabolites (25 nM to 1 mM) were titrated against indicated concentrations of...
labeled proteins and centrifuged for 5 min at 13000 x g. The MST analysis was performed in Monolith NT.115 glass capillaries using the Monolith NT.115 Red/Green device (NanoTemper, Munich) with 20 % LED Power and 40 % MST Power. The overall affinity (K_D) was determined using the NanoTemper Analysis Software.

Relative molecular mass estimation by size exclusion chromatography

Proteins were analyzed on a Superdex 200 increase 3.2/300 SEC chromatography column (GE Healthcare) in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4. The column was calibrated using 20 μg of gel filtration standards (Thyroglobulin, Mr 669,000; void volume; Ferritin, Mr 440,000; Aldolase, Mr 158,000; Conalbumin, Mr 75,000; Ovalbumin Mr 44,000) (GE Healthcare). The relative molecular masses of the peaks obtained were used to generate a calibration curve using a logarithmic interpolation.

Analysis of proteins by PAGE and immunoblotting

Purified protein samples were separated on 12% SDS-polyacrylamide gels. To analyze oligomerization, the recombinant protein was analyzed by a 5-10% gradient PAGE under non-denaturing conditions, lacking SDS or reducing agents and using 750 mM e-Aminocapronsäure and 5% (w/v) Coomassie G 250 as loading buffer. NativeMarker™ was obtained from Life Technologies, Carisbad, CA.

Chlorophyll Extraction

Chlorophyll determination of Arabidopsis leaves was performed following the method described by (24). A total of 250 mg of leaf tissue was harvested and incubated in 2 ml of dimethylformamide for 2 h in the dark. Absorbance was measured at 663, 750, and 645 nm. Chlorophyll concentration was calculated as described (25).

Protein sequence analysis

Protein secondary structure predictions was performed with PSSPred (https://zhanglab.ccmb.med.umich.edu/PSSpre d) (26). BLAST searches were performed with the National Center for Biotechnology Information database (27). All sequences are available from GenBank (http://www.ncbi.nlm.nih.gov). Accession numbers are as follows: STY8 (At2g17700), STY17 (At4g35708) STY46 (At4g38470), SSU (AAA34116).

Quantification and statistical analysis

Quantification of kinase assays was performed with ImageQuant software (GE Healthcare). Student t-tests were performed as indicated.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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ABBREVIATIONS

SAM, s-adenosylmethionine; Ile, isoleucine; MST, microscale thermophoresis; SSU, ribulose-1,5-bisphosphatase; SEC, size exclusion chromatography.
Figure 1: Deletion of the ACT domain enhances STY kinase activity and influences substrate binding.

A) *In vitro* phosphorylation assay with *E. coli* purified WT STY8, STY17, and STY46 and ACT domain deleted variants of each kinase, as well as non-functional kinases. Top panels show autophosphorylation of the kinases, bottom panels show pSSU substrate phosphorylation. A coomassie blue gel shows equal loading of proteins (CBB). For quantification of the phosphorylation assays the WT kinase phosphorylation yields were set to 100%, data show mean ± s.e.m.; n = 3. Asterisks indicate statistical significance as compared to WT control.

B) Binding affinities of STY8 variants and pSSU. MST was performed with STY8, STY8ΔACT and STY8 T439A mutants. Concentrations from 0.1 nM to 3 μM of the kinases were titrated against fluorescence labeled pSSU (25 nM). Weakest interaction was observed between pSSU and STY8 T439A mutant when compared to WT STY8 (K<sub>D</sub> of 708 ± 6.5 nM nM and 8.08 ± 1.4 nM, respectively). Strongest interaction was observed between pSSU and STY8ΔACT mutant (K<sub>D</sub> = 4.20 ± 2.5 nM), n = 3.
Figure 2: STY8 and the STY8-ACT domain form oligomers in vitro. Elution profiles after SEC of with purified STYkinase. A) Elution profile of STY8. B) Elution profile of STY8-ACT. The size of the major peaks is indicated with a triangle was calculated to be 217 kDa (A) and 35 kDa (B). C) PAGE analysis with recombinant STY8 under non-denaturing (Native-PAGE) and denaturing (SDS-PAGE) conditions. Amounts of recombinant protein loaded are given in µg. Gels were either stained with Coomassie.
Figure 3: STY8 is negatively regulated by Ile and SAM via the ACT domain. A) Quantification of kinase assays comparing the activity of 0.25 µg E.coli purified STY8 in presence of 5 mM of the indicated amino acids. Significant decrease in autophosphorylation was only observed in presence of Ile. For quantification of the phosphorylation assays the WT kinase phosphorylation yields were set to 100%, data show mean ± s.e.m.; n = 3. Asterisks indicate statistical significance as compared to untreated control. B) In vitro kinase assay comparing the autophosphorylation of recombinant STY8 WT or STY8ΔACT in presence of increasing concentrations of Ile (1, 2 and 5 mM). WT STY8 sensitivity to Ile is increased in a concentration dependent manner in STY8, but not in STY8ΔACT. A coomassie blue gel shows equal loading of proteins (CBB). C) Ile binds to STY8 via the ACT domain. The direct binding of Ile to STY8 and STY8ΔACT was assessed by MST. The kinases were labeled with Tris-NTA dye. The concentration of the labeled STY8 and its variant was held constant at 25 nM while Ile was titrated in 2-fold serial dilutions against it and the thermophoretic mobility was monitored. Binding was observed between STY8 and Ile with a K_d of 311.4 ± 16 µM. No Ile binding was observed using STY8ΔACT. D) In vitro kinase assay comparing the autophosphorylation of recombinant STY8 WT or STY8ΔACT in presence of increasing concentrations of SAM (1, 2 and 5 mM). WT STY8 sensitivity to SAM increased in a concentration dependent manner. A coomassie blue gel shows equal loading of proteins (CBB). E) The direct binding of SAM to purified STY8 and STY8ΔACT was assessed by MST as in (C). Binding was observed between WT STY8 and SAM with a K_d of = 774.7 ± 39 µM. No SAM binding was observed using STY8ΔACT. F) SAM, Ile and Methionine binding to the STY8 ACT domain by MST. Binding was observed between STY8-ACT and SAM (47.28 ± 25 nM) as well as Ile (81.48 ± 34 µM). No binding constant could be calculated for Methionine. G) SAM and Ile influence pSSU binding to STY8. MST with STY8 and pSSU was performed in presence of Ile and/or SAM. pSSU was labeled with Tris-NTA dye. The final pSSU and SAM and/or Ile was held constant at 25 nM and 1 mM, respectively. STY8 was titrated in 2-fold serial dilutions against it and the thermophoretic mobility was monitored. H) In vitro kinase assay comparing the autophosphorylation of recombinant STY8, STY17, STY46 WT or STY8ΔACT, STY17ΔACT or STY46ΔACT in presence of Ile or SAM (5 mM). A Coomassie blue gel shows equal loading of proteins (CBB). For quantification of the autophosphorylation STY8, STY17 or STY46 WT kinase activity without addition of metabolites was set to 100%, data show mean ± s.e.m.; n = 3. Asterisks indicate statistical significance as compared to untreated control.
Figure 4

A

Ile (mM)

B

Ile (mM)

STY8

STY8 G197D

STY8ΔACT

α2

α1

β1

β3

β2

βαβαβα

C

SAM (mM)

D

ΔF_{norm}[%]

Ile (nM)

ΔF_{norm}[%]

SAM (nM)
Figure 4: A Conserved glycine in the ACT domain is important for Ile binding.  

A) Predicted structure of the ACT domain of STY8 in comparison with the schematic fold typical for ACT domains. The α-helices, β-sheets and the conserved glycine of the presented protein domain structures is denoted in red, blue, and green, respectively.  

B) Kinase assay comparing the autophosphorylation of STY8 WT, STY8ΔACT and STY8G197D mutants in presence of increasing Ile concentrations. WT STY8 shows concentration dependent sensitivity to Ile whereas STY8G197D is insensitive to Ile, comparable to STY8ΔACT. A Coomassie blue gel shows equal loading of proteins (CBB). For quantification of the autophosphorylation WT kinase activity without addition of Ile was set to 100%, data show mean ± s.e.m.; n = 3. Asterisks indicate statistical significance as compared to untreated control.  

C) Kinase assay comparing the autophosphorylation of STY8 WT, STY8ΔACT and STY8G197D mutants in presence of increasing SAM concentrations. WT STY8 and STY8G197D show equal concentration dependent sensitivity to SAM. A Coomassie blue gel shows equal loading of proteins (CBB). For quantification of the autophosphorylation WT kinase activity without addition of Ile was set to 100%, data show mean ± s.e.m.; n = 3. Asterisks indicate statistical significance as compared to untreated control.
Figure 5

(A) Western blots showing the expression levels of STY8, STY46, and RCE1 in different genotypes.

(B) mRNA levels of sty8 sty46, sty8 sty46 35S::STY46, and sty8 sty46 35S::STY46 ΔACT genotypes.

(C) Phosphorylation of pSSU in plant lysate using CBB staining.

(D) Phosphorylation yield of pSSU in different genotypes.

(E) Chlorophyll levels in Col-0, sty8 sty46, sty8 sty46 35S::STY46, and sty8 sty46 35S::STY46 ΔACT genotypes with control and SAM + Ile treatments.

(F) Images showing the phenotypic differences between Col-0 and sty8 sty46 genotypes.
Figure 5: Expression of 35S::STY46 and 35S::STY46ΔACT in WT (Col-0) and sty8 sty46 background in Arabidopsis. A) Expression analysis of STY8 and STY46 by RT-PCR the generated Arabidopsis mutants. RCE1 was amplified as a positive control. B) Quantitative RT-PCR of STY8 and STY46. Values were calculated relative to housekeeping gene RCE1 and expression levels relative to the WT are given (n = 3). C) Soluble protein extract isolated from WT (Col-0), sty8 sty46, sty8 sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT was used to phosphorylate pSSU. A Coomassie stained gel in shown to depict equal loading (CBB). Quantification of the autoradiogram is shown below, data show mean ± s.e.m.; n = 3. Asterisks indicate statistical significance. D) Greening analysis in WT (Col-0), sty8 sty46, sty8 sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT. Three representative seedlings are shown 4 h after illumination. The lower panel shows seedlings grown on MS plates supplemented with 8 µM SAM and 100 µM Ile. E) Chlorophyll concentration was measured 4 h after exposure of etiolated seedlings to light. Plants were either grown on MS plates (control) or on MS plates supplemented with SAM and Ile (SAM + Ile), n = 3. Chlorophyll concentration was measured in µg/mg fresh weight. Data show the mean ± s.e.m.; n = 3. Asterisks indicate statistical significance in samples compared as indicated. F) Phenotype of WT (Col-0), sty8 sty46, sty8 sty46/35S::STY46 and sty8 sty46/35S::STY46ΔACT after growth on soil for three weeks under long day conditions. Scale bars represent 2 cm. No significant difference was found comparing sty8 sty46/35S::STY46ΔACT and WT.
Figure 6: Schematic presentation of the regulation of STY kinase activity by the ACT domain. Predicted structures of the STY8 ACT domain as well as kinase domain are present. Autophosphorylation is represented by a green ‘P’. Different intensities of green refer to etioplast to chloroplast transition efficiency. See text for details.
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Correction: The ACT domain in chloroplast precursor–phosphorylating STY kinases binds metabolites and allosterically regulates kinase activity.
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The abbreviation for aspartate kinase-chorismate mutase-tyrA (prephenate dehydrogenase) was omitted. The abbreviation is ACT. This error has been corrected in the HTML version of this article.