TOUSLED Is a Nuclear Serine/Threonine Protein Kinase That Requires a Coiled-coil Region for Oligomerization and Catalytic Activity*

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The TOUSLED (TSL) gene is essential for the proper morphogenesis of leaves and flowers in Arabidopsis thaliana. Protein sequence analysis predicts TSL is composed of a carboxyl-terminal protein kinase catalytic domain and a large amino-terminal regulatory domain. TSL fusion proteins, expressed in and purified from yeast, were used to demonstrate TSL protein kinase activity in vitro. TSL trans-autophosphorylates on serine and threonine residues, and phosphorylates exogenous substrates. Using the yeast two-hybrid system, TSL was found to oligomerize via its NH2-terminal domain. A deletion series indicates that a region containing two α-helical segments predicted to participate in a coiled-coil structure is essential for oligomerization. TSL localizes to the nucleus in plant cells through an essential NH2-terminal nuclear localization signal; however, this signal is not necessary for protein kinase activity. Finally, deletion mutants demonstrate a strict correlation between catalytic activity and the ability to oligomerize, arguing that activation of the protein kinase requires interaction between TSL molecules.

During development of many organisms, protein kinases function in signaling pathways important for proper morphogenesis and cell fate determination. Their activity can either be stimulatory, such as that of Sevenless receptor kinase (reviewed in Ref. 1), or inhibitory, as exemplified by the inactivation of CAMP-dependent protein kinase through the Hedgehog cascade (reviewed in Ref. 2). Therefore, to understand the basic cellular processes involved during development requires knowledge of how the relevant kinase(s) is regulated, as well as how its target substrates are affected by phosphorylation.

The TSL gene was identified by mutational analysis in Arabidopsis thaliana as a gene required for proper development of the flower and the margins of the leaf (3). tsl loss of function mutations are recessive and cause a phenotype characterized by two major floral defects. First, there is a stochastic decrease in the number of floral organs, implying that TSL functions at an early stage during flower formation, perhaps regulating the establishment of organ primordia by promoting specific cell divisions within the floral meristem. Second, specific regions of the ovule-housing organ, the gynoecium, fail to develop properly suggesting that TSL also may function to pattern developmental programs within an organ type (3).1

TSL encodes a 688-amino acid protein (TSL) which is a putative serine/threonine protein kinase with a COOH-terminal catalytic domain (amino acids 409–688) and an NH2-terminal domain (amino acids 1–408) of unknown function (3).2 Recent data base searches suggest that TSL is a member of an evolutionarily conserved protein kinase subfamily with closely-related homologs found in Caenorhabditis elegans, Caenorhabditis briggsae, humans, and maize.3 The presence of TSL homologs in both plant and animal kingdoms implies the protein performs a fundamental function. Consistent with this hypothesis, TSL is expressed in all organs of the plant (3).

The utilization of the same signaling components in multiple developmental pathways is emerging as a common theme in many organisms (1, 4–7). The requirement for TSL function at several stages of development suggests that this putative protein kinase is regulated in response to different unidentified developmental cues. The TSL NH2-terminal domain contains sequence motifs, such as a coiled-coil region and three consensus nuclear localization signal (NLS) sequences, which together could participate in modulating the activity of the COOH-terminal catalytic domain. The existence of multiple NLS sequences in the NH2-terminal domain may direct the subcellular localization of the protein, permitting access to potential regulatory factors and target substrates. The coiled-coil region, including a leucine-zipper motif, may participate in protein-protein interactions that affect kinase activity. Such interactions could include the formation of TSL oligomers. The ligand-binding induced dimerization of receptor protein kinases is known to be critical for kinase activation (8, 9). However, oligomerization has only recently been found to provide a possible means of regulation of non-receptor protein kinases (10, 11).

To begin to understand the basic properties of the TSL protein kinase and its regulation, we have isolated catalytically active TSL from yeast and characterized that activity in vitro. In this report, we show that TSL is a nuclear serine/threonine protein kinase and is capable of autophosphorylation in trans. The TSL NH2-terminal domain also is shown to mediate oligomerization in the two-hybrid system. Deletion mutants, analyzed for both their enzymatic activity in vitro and ability to

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1 Roe, J. L., Nemhauser, J. L., and Zambryski, P. C. (1997) Plant Cell, in press.
2 J. L. Roe, unpublished observation.
3 The abbreviations used are: NLS, nuclear localization signal; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; MBP, myelin basic protein; GUS, β-glucuronidase; DBD, DNA-binding domain.

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oligomerize in the two-hybrid system, indicate that TSL requires self-association for protein kinase activity.

**EXPERIMENTAL PROCEDURES**

**Strains**

_Escherichia coli_ DH5α (F-, recA1, endA1, hsdR17, supE44, thi1, gyrA, relA1) was the recipient for all plasmid transformations. For drug selections, LB media was supplemented with carbenicillin (100 μg/ml). The yeast strain Y153 (12) (MATα leu2-3,112 ura3-52 trpl-901 his3-200 ade2-101 gal4Δ gal80Δ URA3 GAL1-1A GAL1-1B LYS2 GAL-HIS3) was used for two-hybrid experiments. BJ5460 (MATα ura3-52 trpl lys2-801 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1B R C1 GAL1) was used for protein expression experiments. Yeast YPD and synthetic complete (SC) media was prepared as described (13).

**Plasmids**

_TSL Subclones—_ Precursor plasmids containing the full TSL coding sequence, and the coding sequence for the NH2-terminal domain, and NH2-terminal deletions were generated for subcloning into the expression vectors described below. Cloning details will be provided upon request.

**K38E Mutation—_ A polymerase chain reaction product containing the sequence for the catalytic domain with the codon for Lys-438 changed to codon for Glu was generated by polymerase chain reaction-directed oligonucleotagites using the mutagenic primer 5'-GTGC-GAGCTTCTAGTGT-3', and the T3 and T7 primers by the method of Bowman et al. (14). The fragment was subcloned into pBluescriptSK+ at the EcoRI site to generate pTK2E. This plasmid was subsequently used to exchange the mutant fragment with wild-type fragments in the precursor plasmids where appropriate.

**GST Fusion Plasmids—_ YEpLG-GST* is a yeast shuttle vector containing a LEU2 and a β-lactamase gene for selection in yeast and _E. coli_, respectively, and a 2μ and a CoE1 origin for replication in yeast and _E. coli_. It also contains the coding sequence for GST under the GAL1 control followed by a polylinker. TSL sequences from precursor plasmids were subcloned into the polylinker region of YEpLG-GST such that the TSL coding sequences were in-frame with GST (details will be provided upon request).

**Two-hybrid Constructs—_** TSL sequences from the precursor plasmids described above were subcloned into the two-hybrid vectors pAS1 (12) and pACTII (15) such that the TSL coding sequences were in-frame downstream of the Gal4 DBD or activation domain sequence, respectively (details will be provided upon request).

**Myctope Tag Expression Plasmids**

The pA6M vector, containing a CaMV35S promoter upstream of the sequence encoding a Myc-epitope tag followed by a polylinker, was constructed by ligating the XhoI-XhoI restriction fragment from pJR1265, containing a translation start followed by a 6x Myc-epitope tag and polylinker, into the plant expression vector, pART7 (16). The sequence for full-length TSL was introduced into pA6M to create pA6M/TSL2 (for this subcloning only, the sequence at the translational start site of TSL was changed to introduce an NcoI site, resulting in a serine to alanine change at the second residue in TSL to create TSL2). Deletion mutants were introduced into pA6M by excising appropriate restriction fragments from precursor plasmids and ligating to pA6M.

**GUS Expression Plasmids—_** pRTL2GUS, an expression plasmid containing the GUS reporter gene under the control of the CaMV35S promoter has been described (17). Sequences encoding amino acids 12–438 of TSL were inserted downstream as a BamHI-KpnI fragment replacing the BglII-KpnI Nia fragment from pRTL2SG-Nia/ΔB+p (17) creating pGUS-NTΔN2.

**Expression and Purification of GST Fusion Proteins**

YEpLG-GST constructs were introduced into BJ5460 by chemical transformation (18). For galactose inductions, transformants were grown overnight in 10 ml of SC media lacking leucine and containing 2% raffinose and 0.2% sucrose. 10 ml of fresh media was added and cells grown an additional 4 h. Galactose was added to 2% and cells grown another 4 h. Cells were pelleted by centrifugation and resuspended in lysis 250 buffer (50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM sodium fluoride, supplemented with phenylmethylsulfonyl fluoride (100 μg/ml), apotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (1 μg/ml), dithiothreitol (1 mM), and benzamidine (1 mM)) prior to freezing. Glass beads were added to thawed samples and cells lysed by vortexing. Extracts were cleared by centrifugation (14,000 x g for 20 min at 4 °C) and protein concentration of supernatants estimated by OD280. Equal protein amounts were mixed with glutathione-agarose beads (Sigma) for 1 h at 4 °C. Bound fractions were washed extensively with lysis 250 buffer. For all purifications, a ratio of 1 μg of protein extract/100 μl bed volume of glutathione-agarose beads was used.

**Western Blot Analysis**

Bound GST fusion proteins from equivalent aliquots of glutathione-agarose beads (10-μl bed volume) purified as above were eluted by boiling in Laemmli sample buffer and separated by SDS-PAGE (19). For Western blots, as in Figs. 2A, 4A, and 5A, the entire sample was loaded, and in Fig. 7A, one-half volume of eluted proteins was loaded. Gels were transferred to Immobilon-P and immunoblots were performed using rabbit anti-GST polyclonal antiserum, horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad), and detection with enhanced chemiluminescent detection. 

**Kinase Assays**

Equivalent aliquots of glutathione-agarose beads (10-μl bed volume) with bound GST fusion proteins purified as above were washed three times in kinase buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 10 mM MgCl2, 2 mM MnCl2) and resuspended in 37.5 μl of kinase buffer. 10 μCi of [γ-32P]ATP (Amersham, 3000 Ci/mmol) was added and the reaction was incubated at room temperature for 60 min. Laemmli sample buffer was added and samples were fractionated for 3 min, centrifuged, and the supernatant was analyzed by SDS-PAGE. Kinase assays in Fig. 7B were washed in kinase buffer after the reaction prior to elution in Laemmli sample buffer. In kinase assays in Figs. 2, B and D, and Fig. 5B, the entire reaction was loaded, whereas in Fig. 7B, one-fourth volume of the reaction was loaded.

**Phosphoamino Acid Analysis**

Aliquots of purified GST-TSL were allowed to autophosphorylate as described above. Pellets were washed once in kinase buffer, samples were boiled after addition of Laemmli sample buffer and electrophorezed on an SDS-polyacrylamide gel. The portion of the gel containing the phosphorylated protein was transferred to Immobilon-P (Amersham) by electroblotting. The 32P-containing portion of the membrane was excised, and the sample was analyzed by incubating the membrane in 50 mM HCl for 1 h at 100 °C (20). The supernatant was dried, redried twice after resuspension in H2O, and analyzed by TLC and autoradiography by the method of Cooper et al. (21).

**Quantitation of β-Galactosidase Activity**

β-Galactosidase activity in yeast was quantified with chlorophenyl red-β-D-galactopyranosidase (CPRG, Boehringer Mannheim). Y153 transformants were grown in 3 ml of SC media lacking tryptophan and leucine to OD600, 1.0–1.5. Cells were then prepared and permeabilized as described (22), except cell pellets were resuspended in 900 μl of H buffer (100 mM HEPES, 150 mM NaCl, 2 mM MgCl2, 1% bovine serum albumin, pH 7.0) and 100 μl of 50 mM chlorophenyl red-β-D-galactopyranosidase was added following permeabilization. The amount of liberated chlorophenyl red was determined by OD574.

**Immunocytochemistry and GST Fusion Assays**

Nicotiana tabacum (line XD) suspension cultures were grown as described (23). Protoplasts were prepared as described previously (24) and electrophorezed using a Bio-Rad electrophoretor according to McLean et al. (23). Cells were then grown at 22 °C in suspension media supplemented with 0.4% mannitol for 16–24 h. For immunofluorescence staining, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) (pH 7.2) for 10 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. All antibody and washing steps were performed in blocking buffer (0.1% Triton-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween 20, 5% (w/v) dry milk). Myc epitope-tagged proteins were detected using the monoclonal antibody, 9E10 (BAbCO), and a goat anti-mouse fluorescein isothiocyanate antibody.
isothiocyanate-conjugated secondary antibody (Calbiochem). Cells were resuspended in Citifluor (Ted Pella, Inc.) prior to visualization. Digital images of fluorosein isothiocyanate or 4,6-diamidino-2-phenylindole fluorescence, or of Normarski differential interference contrast, were obtained using a Zeiss Axiophot fluorescence microscope with a CCD camera. GUS staining was performed as described previously (24). When color developed, cells were photographed by bright-field illumination on a Zeiss Axiophot.

RESULTS

TSL Domain Structure and Deletion Constructs—The COOH-terminal domain of TSL contains the consensus sequences shared by the large family of serine/threonine and tyrosine protein kinases (3) (Fig. 1). In addition to the COOH-terminal catalytic domain of TSL, the primary structure of the NH2-terminal domain contains several regions which, by homology to other proteins, may act as functional domains. These regions are depicted in Fig. 1, and include a glutamine repeat, three putative NLS, and a coiled-coil region containing a leucine-zipper motif. The site of the point mutation in tsl-2 allele (Val-355 to Asp). K438E represents the position of the mutant generated in the conserved Lys-438 to Glu. Numbers represent amino acid numbers. Deletions are diagrammed below where NT, NH2-terminal domain, Δc/e, deletion of coiled/coil region; and ΔNn represents deletions of NH2-terminal sequences to amino acid number n.

Purified TSL and K438E fusion proteins were used in kinase assays to determine possible catalytic activity. Uninduced cell extracts also were mock-purified and shown to contain no contaminating protein kinase activity (Fig. 2B, lanes 1 and 2). Purified TSL fusion protein autophosphorylates as evidenced by [32P]phosphate incorporation into the 100-kDa protein (Fig. 2B, lane 3) identified as TSL (Fig. 2A, lane 3). In contrast, the mutant K438E (Fig. 2B, lane 4) or pre-boiled TSL (Fig. 2B, lane 8) show no incorporation. The autophosphorylation of TSL may account for the slight decrease in mobility compared with that of mutant K438E (Fig. 2A, compare lanes 3 and 4). TSL autophosphorylation activity is dependent on either of the divalent cations Mg2+ or Mn2+ (Fig. 2B, lanes 5–7).

Phosphoamino acid analysis was performed on purified TSL fusion protein which had undergone autophosphorylation (Fig. 2C). [32P]Phosphate is incorporated into both phosphothreonine and phosphoserine. Precise mapping of the autophosphorylation sites in TSL will be necessary to determine the number and location of modified residues. Control experiments showed that no [32P]phosphate was incorporated into the GST portion of the autophosphorylated TSL fusion protein (data not shown).

Purified GST-TSL protein kinase was tested for the ability to phosphorylate exogenous substrates. Both myelin basic protein (MBP) (Fig. 2D, lane 1) and casein (Fig. 2D, lane 5) were phosphorylated by TSL, whereas histones (Type IIIS, including histone H1) were not (Fig. 2D, lane 3). Purified GST-K438E showed no activity on added proteins (Fig. 2D, lanes 2, 4, and 6).

TSL Localizes to the Plant Cell Nucleus—Having established TSL is an authentic protein kinase, next we analyzed the
potential functions of the \( \text{NH}_2 \)-terminal domain. The TSL sequence contains three putative NLS, all in the \( \text{NH}_2 \)-terminal domain (Fig. 1). To determine the subcellular localization of TSL in plant cells, the TSL coding sequence was fused to sequences encoding a Myc-epitope tag present in a plant expression vector, pA6M. In this vector, expression of tagged proteins is under the control of the constitutive CaMV35S promoter. The A6M.TSL-2 plasmid was electroporated into tobacco protoplasts, and cells incubated for 18–24 h to allow expression of the fusion protein. Indirect immunofluorescence was performed using the anti-Mycin monoclonal antibody, 9E10 (29). The Myc-TSL fusion protein is detected exclusively in the nucleus of tobacco cells (Fig. 3, A and B). To determine if the \( \text{NH}_2 \)-terminal domain is sufficient to direct nuclear localization in plant cells, sequences encoding amino acids 12–438 of TSL were fused in-frame downstream of the \( \beta \)-glucuronidase (GUS) reporter gene to create pGUS-NTA\( \Delta \)N12. When GUS alone is expressed in tobacco protoplasts, enzymatic activity is found only in the cytoplasm (Fig. 3D). When the GUS-NTA\( \Delta \)N12 fusion protein is expressed in protoplasts, GUS activity is nuclear (Fig. 3C), indicating that the \( \text{NH}_2 \)-terminal domain of TSL is sufficient for nuclear targeting of the protein. Together, these results demonstrate that TSL is a nuclear protein, and that one or more of the \( \text{NH}_2 \)-terminal NLS motifs likely confers localization.

The Coiled-coil Region Is Required for TSL Oligomerization—The \( \text{NH}_2 \)-terminal domain of TSL contains two segments predicted to form \( \alpha \)-helices which each may participate in a coiled-coil structure (30) (cf and cII in Fig. 1). The second of these segments contains a leucine-zipper motif. By analogy to other proteins, this coiled-coil region may be involved in protein-protein interactions (30, 31). To test if TSL can self-associate via either of these \( \alpha \)-helical segments, the yeast two-hybrid system (32) was used (Fig. 4). Full-length TSL could not be used in these experiments, as a construct containing the NH\( \text{NH}_2 \)-terminal terminus fused to the Gal4 DNA-binding domain (DBD) weakly activates the GAL1 promoter (data not shown). However, the near full-length deletion \( \Delta \)N73-DBD which lacks the glutamine repeat, does not activate the promoter, allowing its use in the assay (Fig. 4A, \( \Delta \)N73-DBD plus no insert control). When \( \Delta \)N73 is co-expressed as both a DBD and an activation domain fusion, the reporter gene is activated (Fig. 4A), indicating that TSL oligomerizes. To identify the region(s) necessary for this interaction, TSL deletions were inserted into both the activation domain and DBD containing plasmids, and tested for self-association as well as for interaction with \( \Delta \)N73. \( \Delta \)N171, with an intact coiled-coil region, retains the ability to interact both with itself and with \( \Delta \)N73 (Fig. 4A). The first \( \alpha \)-helical segment and part of the second is removed (\( \Delta \)N304), however, oligomerizing activity is lost, even though the full leucine-zipper is still present. All further deletions, including \( \Delta \)N403 which retains the catalytic domain, face to self-oligomerize or interact with \( \Delta \)N73 (Fig. 4A). Thus, the first \( \alpha \)-helical segment of the coiled-coil region is apparently essential for TSL oligomerization.

Further evidence for the involvement of the coiled-coil region
in TSL oligomerization was found by using deletion mutants lacking the catalytic domain in the two-hybrid assay (Fig. 4B). When the entire NH2-terminal domain (NT) (amino acids 1–406) (activation domain fusion) is co-expressed with the NH2-terminal domain deletion NTΔN73 (amino acids 73–406) (DBD fusion), a strong interaction is observed, indicating that the NH2-terminal domain alone can self-associate (Fig. 4B). NTΔc, an internal deletion which removes most of the coiled-coil region (from amino acids 172–327), fails to interact with NTΔN73 confirming the importance of this region in mediating oligomerization (Fig. 4B). Interestingly, when NTΔN73 association with ΔN73 (containing the catalytic domain) was tested, no interaction is observed (Fig. 4B). Thus, the presence of the catalytic domain in only one of the partners in some way precludes the formation of heterotypic oligomers. Alternatively, the NH2-terminal domain alone may adopt a different conformation that prevents interaction with full-length TSL. Nevertheless, the results indicate that TSL can oligomerize, and that this ability is dependent on an intact coiled-coil region.

Catalytic Activity of Deletion Mutants Correlates with Ability to Oligomerize—The deletion mutants next were expressed and purified as GST fusions from yeast and tested for their ability to autophosphorylate and to transphosphorylate MBP. Western blot analysis of purified fusion proteins probed with anti-GST antiserum showed that all the deletion mutants are synthesized at similar levels (Fig. 5A). Kinase assays show that the two mutants capable of oligomerization, ΔN73 and ΔN171, also are both capable of autophosphorylation and transphosphorylating MBP (Fig. 5B, lanes 2 and 3, respectively) at levels comparable to wild-type. Deletions affecting the coiled-coil region, however, result in catalytically inactive fusion proteins (Fig. 5B, lanes 4–7). These results imply that the NH2-terminal domain in some way imparts a positive effect on the activity of the catalytic domain. The strict correlation between kinase activity and oligomerization seen with these mutants strongly suggests that oligomerization mediated by the coiled-coil region is required for TSL activity.

Subcellular Localization of TSL-deletion Mutants in Plant Cells—The two catalytically active TSL-deletion mutants, ΔN73 and ΔN171, differ in the NLS consensus sequences they retain. ΔN73 contains all three putative NLS sequences (see Fig. 1). The first and third NLS consensus sequences are SV40 T-antigen-type signals as defined by Chelskey et al. (33), whereas the second is a putative bipartite signal (34). ΔN171 lacks the first and second putative NLS sequences, but retains the third potential NLS near the catalytic domain. To test if the subcellular distribution of ΔN73 and ΔN171 differs, a Myc-epitope tag was added at their respective NH2 termini and fusion proteins were transiently expressed in tobacco protoplasts. Nuclei were visualized by 4,6-diamidino-2-phenylindole staining (Fig. 6, B and E) and bright-field imaging (Fig. 6, C and F). The epitope-tagged TSL mutants were localized by indirect immunofluorescence using the 9E10 monoclonal antibody (Fig. 6, A and D). As expected, ΔN73 is nuclear-localized (Fig. 6A), as is the full-length protein (Fig. 3A). However, ΔN171 is excluded from the nucleus in expressing cells, and is found in the cytoplasm which surrounds the nucleus and the vacuole (Fig. 6D), indicating the third putative NLS is not sufficient to direct the protein to the nucleus. These results also

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**FIG. 4.** TSL can oligomerize in the two-hybrid assay and this interaction requires the first α-helical segment of the coiled-coil region. TSL deletions were joined to the carboxyl terminus of the Gal4 DBD and/or activation domain as indicated. The yeast strain, Y153, was transformed with the plasmid combinations shown and transformants assayed for β-galactosidase activity. Numbers are expressed as Miller units and represent the average from three independent transformants. A, on the left, the deletions used for activation domain fusions are diagrammed. These were tested with either the same deletion as a DBD fusion (right) or with ΔN73-DBD (left). B, on the left, the deletions used for activation domain fusions are diagrammed. These were tested for interaction with NTΔN73-DBD.

**FIG. 5.** Protein kinase activity of TSL deletion mutants correlates with their ability to oligomerize. A, Western blot analysis of purified TSL (lane 1) and NH2-terminal deletion GST fusion proteins (lanes 2–7) using anti-GST antiserum. Equivalent amounts of protein extract were purified for each sample. B, kinase assays of TSL (lane 1) and NH2-terminal deletion GST fusion proteins (lanes 2–7). 1 μg of MBP was added before addition of [γ-32P]ATP. After the reaction, Laemmli sample buffer was added, samples were boiled, and proteins were separated on a 12.5% SDS-polyacrylamide gel. The autoradiogram shows two different regions of the gel. The arrow indicates the position where MBP migrates. The results presented are representative of two independent experiments.
suggest that nuclear localization is not necessary for activation of the catalytic domain as the ΔN171 protein is an active protein kinase in vitro. Smaller, catalytically inactive deletion mutants, including ΔN304 and ΔN403, were found in both the cytoplasm and nucleus presumably because the molecular mass of the mutant proteins is at or below the reported size exclusion limit of the nuclear pore complex (~40 kDa, reviewed in Ref. 35) (data not shown).

Trans-phosphorylation Occurs in TSL Complexes—In many cases autophosphorylation of a protein kinase has been shown to be an intramolecular phosphorylation event (reviewed in Ref. 36). To test if TSL trans-autophosphorylates, extracts containing the kinase-dead mutant K438E were mixed and copurified with ΔN171, the largest active deletion mutant that clearly resolves from the full-length K438E by SDS-PAGE (Fig. 7A). As expected, purified GST-ΔN171 is active (Fig. 7B, lane 1), whereas GST-K438E is not (Fig. 7B, lane 2). A kinase assay of the copurified samples reveals that ΔN171 can transphosphorylate K438E (Fig. 7B, lane 3), and K438E is apparently comparable to the wild-type protein as a substrate. This result demonstrates that autophosphorylation of TSL is, at least in part, a trans-phosphorylation event. Precise mapping of the phosphorylation sites on both the active and inactive kinases will be necessary to determine if some sites modified during autophosphorylation also are due to intramolecular events.

To ask if catalytic activity is required for TSL oligomerization, we tested the interaction ability of the K438E mutant in the two-hybrid system (Fig. 7C). ΔN73-K438E can self-associate (Fig. 7C) and activates the lacZ reporter gene to a similar level as the wild-type homotypic interaction (15.8 versus 23.8 Miller units, see Fig. 4A). This indicates that TSL protein kinase activity is not necessary for self-oligomerization, as earlier suggested by the interaction observed between TSL truncations lacking the catalytic domain (Fig. 4B). Interestingly, when tested with ΔN73 and ΔN171, the ΔN73-K438E protein showed a much stronger interaction than either of these wild-type proteins with themselves (Fig. 7C, and see Fig. 4A). This could imply that reciprocal autophosphorylation events weaken the stability of the wild-type TSL complex. However, if this were so, it would be expected that the K438E homotypic interaction would also be stronger than wild-type, which is not observed (see above). Also, ΔN73-K438E displays a weak interaction with NTΔN73 (Fig. 7C) unlike wild-type ΔN73 (Fig. 4B). This observation is confirmed by qualitative β-galactosidase assays (data not shown). This result suggests that the presence of an inactive (or unphosphorylated) catalytic domain affects the conformation of the NH₂-terminal domain to a lesser extent than wild-type (see above). In summary, TSL can phosphorylate and form a heterotypic complex with K438E, and this complex is apparently more stable than either homotypic interaction.

DISCUSSION

The loss of TSL gene function results in a mutant phenotype affecting both leaf and flower morphology, and TSL appears to function during both early and late stages of flower development (3). These results suggest that the TSL gene product participates in a commonly used developmental pathway. Sequence analysis predicted TSL to be a serine/threonine protein kinase composed of a carboxyl-terminal catalytic domain and a large amino-terminal regulatory domain. We have shown here that TSL is indeed a protein kinase which autophosphorylates on both serine and threonine residues. That a kinase-dead mutant can serve as a substrate for the wild-type protein demonstrates that some, if not all, sites are phosphorylated in trans. Furthermore, TSL can phosphorylate exogenous substrates such as MBP and casein in vitro.

TSL function presumably is tightly controlled during Arabidopsis development. We have shown that the TSL NH₂-terminal domain contributes at least two regulatory activities to the
protein. First, this domain contains three NLS sequences, at least one of which appears to be functional in targeting TSL to the plant cell nucleus. Deletion of the first two NLS consensus sequences results in a cytoplasmically localized protein, suggesting that one or both of these NLS sequences are essential for nuclear localization. The third NLS is not sufficient for nuclear targeting. Catalytic activity is not required for the nuclear localizing function as the NH2-terminal domain alone efficiently targets a heterologous protein to the plant cell nucleus. Reciprocally, the deletion mutant ΔN171 is not nuclear-localized, but is catalytically active in vitro, suggesting that nuclear localization is not required for catalytic activity.

The second regulatory function of the NH2-terminal domain is to mediate TSL oligomerization. Deletions studies indicate that at least the first a-helical segment of the coiled-coil region is critical for TSL oligomerization. The leucine-zipper motif found in the second a-helical segment is not sufficient for oligomerization, and its contribution to TSL function is not yet clear. Importantly, deletion mutants revealed a strict correlation between oligomerization in the two-hybrid system and the ability to both autophosphorylate and transphosphorylate exogenous substrates in vitro. That the catalytic domain of TSL alone is inactive indicates that the NH2-terminal domain must positively regulate the protein. Together, these data strongly suggest that oligomerization of TSL is required for activation of the catalytic domain. Although this interaction likely is due to direct binding of TSL molecules, it is possible that a cofactor present in the yeast cell mediates this apparent self-interaction.

The kinase-dead K438E mutant can interact with itself and wild-type TSL, demonstrating that catalytic activity is not required for oligomerization. Based on values obtained for activation of the reporter gene in the two-hybrid assay, K438E apparently interacts with wild-type TSL more strongly than either protein interacts with itself. Perhaps this heterotypic combination represents an intermediate state where autophosphorylation of only one molecule in a complex has occurred and this intermediate is more stable. Functionally, it is not yet known whether a heterotypic complex is catalytically active. If not, the K438E mutant would be expected to exert a dominant negative effect when expressed in wild-type plants. In contrast, the NH2-terminal domain alone (NTΔN73) cannot interact with near full-length TSL containing the catalytic domain (ΔN73) and therefore, presumably will not function as a dominant-negative mutation. Experiments are underway to test these hypotheses.

Ligand-mediated dimerization is generally required for activation of receptor protein kinases (reviewed in Refs. 8 and 9). However, oligomerization is not a common mechanism of regulation for a non-receptor kinase, such as TSL. Recent results suggest that oligomerization plays a role in Raf-1 kinase activation (10). Several other cytoplasmic protein kinases, including double-stranded RNA-dependent protein kinase (37) and Type I and Type II cGMP-dependent protein kinases (38, 39), have been shown to exist as dimers, although the functional relevance of dimerization is not clear. Studies with double-stranded RNA-dependent protein kinase deletion mutants demonstrated that the catalytic domain alone, although unable to dimerize, was fully active in vivo (40). In the case of the cGMP-dependent protein kinases isoforms, it is not known if dimerization is required for catalytic activity. Finally, the crystal structure of the regulatory domain of Lck, a Src family member, revealed that this domain dimerized, but again, it is unknown whether dimerization plays a role in regulating kinase activity (41).

The apparent dependence of TSL catalytic activity on oligomerization argues that TSL self-association via the NH2-terminal domain transmits a conformational change to the catalytic domain, analogous to the effect of dimerization on receptor tyrosine kinases (reviewed in Ref. 9). Dimerization per se is insufficient for activation, as GST can dimerize (42), and therefore, even the catalytically inactive GST-TSL deletion mutants may exist as dimers. TSL self-association mediated by its coiled-coil region, then, may not act merely to bring the catalytic domains into proximity with one another, but instead may cause the protein to adopt a highly specific structure. This conformational change may either allow the catalytic domain to take on a fully active structure, or permit an intermediate that allows one TSL molecule to activate another via transautophosphorylation. This is in contrast to other examples where activation of a protein kinase can be induced by fusion to a heterologous dimerization domain (Refs. 10 and 11, and reviewed in Ref. 8), including fusion to GST (43). However, we cannot exclude the possibility that TSL activation requires complexes larger than dimers, and thus dimerization via GST might be insufficient for activation.

How, then, might TSL oligomerization be controlled? Two obvious possibilities exist. First, the protein may spontaneously form oligomers with a subsequent modification(s) serving as the rate-limiting step for kinase activation. Second, the protein could normally adopt a structure that is unable to oligomerize. An activating event, such as ligand-binding or phosphorylation of a critical residue(s), would then occur to allow TSL self-association. In this scenario, oligomerization could be rate-limiting and/or may require additional modifications for kinase activation. Precedence for the latter mecha-
nism comes from the ligand-mediated dimerization of receptor protein kinases known to be critical for their activation (reviewed in Refs. 8 and 9). If such an activation mechanism is used by TSL, analogous activating components must also be present in yeast.

Active TSL proteins have the ability to autophosphorylate, and as mentioned, trans-autophosphorylation may activate recipients TSL molecules. In this regard, protein kinases can be divided into two broad groups, the RD and non-RD kinases (36). RD kinases, including all tyrosine kinases and many serine/threonine kinases contain an arginine immediately preceding the catalytic aspartate (subdomain VIb). A subset of RD kinases require phosphorylation of an amino acid within the “activation segment,” a region between the conserved DFG (subdomain VII) and XPE (subdomain VIII), for catalytic activity (reviewed in Ref. 36; subdomains from Ref. 44). TSL is a non-RD kinase, however, as the amino acid preceding the conserved catalytic aspartate is a tyrosine (Tyr-538). Of those studied, non-RD kinases do not require modification in the activation segment. Therefore, we assume that if autophosphorylation is required for activation in TSL, a different region is involved. Precise mapping and mutation of the autophosphorylation sites is currently underway to address this issue.

Loss-of-function tsl mutations affect several aspects of plant development, arguing the protein kinase may play different roles in specific tissues and during different stages of development. Input from the various signaling pathways could modulate TSL function by affecting its oligomerization state and therefore, the catalytic activity of the protein. Identification of signals that regulate TSL and the relevant downstream, presumably nuclear, substrates is essential. Recent genetic studies have shown that TSL may interact with several proteins involved in the control of floral organ number and regional specification during gynoecium development. These include ETTIN (45), PERIANTHIA (46), and LEUNIG (47). The availability of catalytically active TSL protein kinase will allow future studies to determine whether these and other potential interactions are direct and if so, how the proteins may regulate one another.

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