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Tnt1 retrotransposon tagging of *STF* in *Medicago truncatula* reveals tight coordination of metabolic, hormonal and developmental signals during leaf morphogenesis

Million Tadege[1,*] and Kirankumar S. Mysore[2]

[1]Institute for Agricultural Biosciences; Oklahoma State University; Ardmore, OK USA; [2]Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, OK USA

Tnt1 **(transposable element if *Nicotiana tabaccum* cell type 1) is one of the very few active LTR retrotransposons used for gene tagging in plants. In the model legume *Medicago truncatula*, *Tnt1* has been effectively used as a gene knock-out tool to generate several very useful mutants. *stenofolia* (*stf*) is such a mutant identified by *Tnt1* insertion in a *WUSCHEL*-like homeobox transcription factor. *STF* is required for blade outgrowth, leaf vascular patterning and female reproductive organ development in barrel medic and woodland tobacco. Using transcript profiling and metabolite analysis, we uncovered that mutant leaves are compromised in steady-state levels of multiple phytohormones, sugar metabolites and derivatives including flavonoids and polyamines. In the *lam1* mutant (caused by deletion of the *STF* ortholog in *Nicotiana sylvestris*), while glucose, fructose, mannose, galactose, myo-inositol and aromatic aminoacids are dramatically reduced, sucrose is comparable to wild-type levels, and glutamine, proline, putrescine, nicotine and sorbitol are highly increased. We demonstrated that both *stf* and *lam1* mutants accumulate reduced levels of free auxin and ABA in their leaves, and ectopic expression of *STF* in tobacco leads to auxin and cytokinin overproduction phenotypes including formation of tumors on the roots and crown. These data suggest that *STF* mediated integration of metabolic and hormonal signals are required for lateral organ morphogenesis and elaboration.**

Keywords: STF, *Medicago truncatula*, lam1, Tnt1, cell division, leaf development, phytohormones, metabolic sugars, homeostasis, signal integration

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*Correspondence to: Million Tadege;
Email: million.tadege@okstate.edu

Long terminal repeat (LTR) retrotransposons are the most abundant mobile genetic elements in plants. Since their mode of transposition is replicative involving an mRNA intermediate, they are very attractive insertional mutagens and can be used as gene tagging tools to saturate relatively large plant genomes such as *Medicago truncatua* which is nearly five times the genome size of *Arabidopsis thaliana*. Most plant LTR retrotransposons, however, are inactive and have become immobile leaving their transposition history behind. *Tnt1* (transposable element if *Nicotiana tabaccum* cell type 1) is one of the very few active plant LTR retrotransposons that has been shown to efficiently transpose in its host tobacco and other plant genomes.[1-4] This ability of *Tnt1* to move in a heterologous host has been exploited to develop a tagged mutant population of over 20,000 lines in the model legume *Medicago truncatula*.[2,5-8] *Tnt1* tagging in Medicago has led to the identification of several mutants affected in diverse plant developmental, metabolic and symbiotic pathways in the last couple of years.[9-13] Here we briefly comment on one of such tagged leaf mutants, *stenofolia* (*stf*), in reference to our recent article published in Plant Cell.[14] The most visible phenotype of the lack of *STF* function is manifested in defective lamina outgrowth, where mediolateral growth is drastically reduced and venation patterning is disrupted.[14] While individual *stf* leaf cell size in width direction is only slightly lower than wild-type, the total number of cells along the equatorial plane of the leaf width is reduced by more than three-fold

suggesting that the *stf* mutant leaf blade has a major defect in cell proliferation. Interestingly, leaf growth in the proximo-distal (length) direction is virtually unaffected prompting the hypothesis that differential regulation of cell division within the same tissue may control leaf size and shape. However, whether the cell proliferation defect is associated with slower rate of cell division or premature exit from the cell cycle is not yet determined. Defects in mediolateral expansion are also manifested in the flower where lateral growth is severely compromised in the outer petal and carpel.

STF has multiple functions critical for plant growth and metabolic signaling including modulating phytohormones and metabolic sugars. The *lam1* mutant in *Nicotiana sylvestrisis* is caused by deletion of the *STF* ortholog, and has a similar leaf phenotype to *stf* albeit stronger. Both *stf* and *lam1* mutants produce reduced steady-state levels of free auxin and ABA.[14] Transgenic *N. sylvestris* plants ectopically expressing *STF* exhibit typical auxin and cytokinin overproduction phenotypes including formation of tumors at the crown and roots. This data suggests that *STF* also regulates cytokinin levels in which lack of *STF* function resulting in auxin and cytokinin deficiency while over-expression of *STF* leading to over-accumulation of these two growth regulating hormones. In agreement with this observation, exogenous application of auxin and cytokinin together to the shoot apex of *lam1* partially rescues the blade phenotype. Both auxin and cytokinin are known to affect cell division and cell expansion, but the exact mechanisms are not fully understood. Microarray analysis of *stf* further indicated that biosynthesis, signaling or homeostasis of several other hormones including ethylene, GA, methyl jasmonate and brassinosteroid is also affected although the magnitude of change remains to be quantified. Interestingly, the requirement of multiple phytohormones and hormonal signal integration at various stages of shoot and leaf development has been highlighted in Arabidopsis.[15-17]

In addition, genes involved in nitrate acquisition and transport, heavy metal detoxification, sugar metabolism including the myo-inositol pathway, drought and salt stress, light perception, polyamine biosynthesis, aromatic aminoacid biosynthesis, the phenylpropanoid pathway including anthocyanin biosynthesis, cell wall biosynthesis including cellulose, lignin and matrix polysaccharides, PDR and ABC type membrane transporters, phospholipid signaling components, as well as epicuticular wax and alkaloid biosynthesis were significantly altered.[14] The exact molecular nature of the association for these gene expression changes with the *STF* activity is yet to be determined, but our analyses so far suggest that most of these changes are specific and are directly or indirectly connected to *STF* function rather than being general consequences of the mutant phenotype. For example, by direct metabolite measurements, we showed that the *lam1* mutant accumulates reduced levels of ABA and auxin, but we have also found that *STF* overexpressors over accumulate ABA and auxin even when the leaves were dwarfed due to overexpression of *STF*. On the other hand, we discovered that the *lam1* mutant over accumulates the diamine putrescine while it has been recently reported that activation of *WOX1* (homolog of *STF*) in Arabidopsis reduces polyamine biosynthesis by directly altering the activity of S-adenosylmethionine decarboxylase (SAMDC) through protein-protein interaction.[18] Since SAMDC is a cytosolic enzyme, this physical interaction suggests localization of the WOX1 protein both in the nucleus and the cytosol. If this function of *WOX1* outside of the nucleus is confirmed in vivo for *STF* as well, it will be an important means of controlling the posttranslational activity of metabolic enzymes in addition to transcriptional regulation. Similarly, transcriptional changes in the myo-inositol pathway and phenylpropaniod pathway enzymes have been associated with specific metabolite changes in the respective pathways suggesting that *STF* is a central coordinator of developmental and metabolic signals.

The molecular mechanism by which *STF/LAM1* integrates metabolic, hormonal and developmental signals remains an open question, and is an active area of investigation in our lab (**Fig. 1**). We hypothesize that the first key to coordination of such global regulation may lie on connecting primary and secondary sugar metabolism to phytohormones via

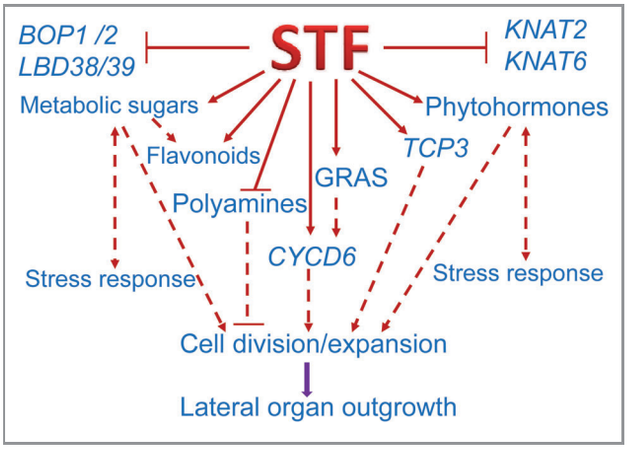

Figure 1. Putative model: *STF* is a central coordinator of metabolic, hormonal and developmental signals. In Medicago leaf primordia, *STF* represses class 1 KNOX and LOB domain transcription factors while activating specific types of GRAS and TCP transcription factors as well as D-type cyclins to promote cell proliferation in the leaf margin controlling blade outgrowth. *STF* also activates sugar metabolism, the phenylpropanoid pathway and phytohormones biosynthesis including auxin, cytokinin, ABA and GA. Metabolic sugars and phytohormones in turn interact with each other and are known to activate cell division and promote growth. *STF* may also regulate stress response indirectly through metabolites such as proline, alanine, glutamine, putrescine or via phytohormones such as ABA. Solid lines indicate our observations in Medicago or tobacco based on a combination of microarray analysis, quantitative RT-PCR, transgenic analysis, hormone measurements and metabolite profiling. Broken lines are inferred by extrapolation from Arabidopsis and other species. Arrows indicate activation while blocked lines indicate repression.

homeostasis, and UDP glucosyl/glucuronosyl transferases and hydrolases may play important roles in this connection. Controlling hormone homeostasis by activating sugar conjugating enzymes when free hormone levels are high and hydrolyzing enzymes when free hormone levels are low is an important and effective means to achieve tissue specificity and local delivery of phytohormones during normal growth as well as during response to environmental cues. However, homeostasis research with respect to hormone biology, plant growth and stress response is yet to gain traction.

A second candidate target for global regulation is the homeostasis of sugar metabolism itself. The amount of sugar in the cytosol and plastids that goes into primary and secondary metabolism is under tight regulation coordinated by sugar signaling, and this is dynamically responsive to developmental and environmental signals. A developmental regulator is, thus, likely to have an effect on sugar homeostasis to coordinate growth and function with environmental adaptation. Alternatively, *STF* may have been selected to recognize the promoters of several genes for direct repression or activation or to recognize several proteins for direct or adaptor mediated protein-protein interactions. Another hypothetical alternative scenario could be that *STF* controls as yet unidentified hormone, for example, the BYPASS1 signal[19,20] that potentially antagonizes the activities of multiple hormones[21] including ethylene, ABA, GA, auxin and cytokinin or the *KLUH/CYP78A5*-dependent growth signal that generates a mobile growth factor.[22-24] Irrespective of the specific molecular mechanisms, it is clear that changes in *STF* activity are directly or indirectly associated with changes that perturb development, metabolism and signaling suggesting that *STF* is a master developmental switch in dicots with far reaching significance to plant morphology and function (**Fig. 1**). Identifying the exact molecular mechanism of *STF* function will enlighten our understanding of how plants coordinate developmental and metabolic signals with their environment.

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