

# LUPIN - A MODEL GRAIN LEGUME SPECIES FOR STUDIES OF TRANSLOCATION AND SIGNALING

Craig Atkins<sup>1</sup>, Penny Smith<sup>2</sup> and Caren Rodriguez<sup>1</sup>

<sup>1</sup>School of Plant Biology University of Western Australia

<sup>2</sup>Biological Science University of Sydney

Corresponding author email: catkins@cyllene.uwa.edu.au

## ABSTRACT

**A number of lupin species are unique among grain legumes in that shallow wounds to their vasculature results in significant ‘bleeding’ of phloem contents, in amounts sufficient for detailed chemical and molecular characterisation. This feature permits separate collection of both xylem and phloem contents as exudates that carry currently formed assimilates and other nutrients, plant growth regulators, peptides and proteins as well as highly specialised nucleic acids (mRNA and si/miRNA) from sites of their synthesis (sources) to sites of unloading and, in the case of signaling molecules, action (sinks). Collections can be made at or close to source organs (e.g. petioles and leaves) as well as at sinks (e.g. flower buds, fruits and stem bases). These tools provide new information about the molecular events that are potentially regulated as a consequence of translocation of solutes, including signaling molecules, and provide a novel approach to understanding assimilate partitioning at this level. While genomic data for lupins is still fragmentary newer sequencing methods and other bioinformatic tools are likely to lead to significant advances in this area in the near future, providing a basis to link translocation data with identification of important genes. Linking translocation and genomic data in lupin will provide a valuable model for other grain legume species.**

## KEYWORDS

lupins, translocation, phloem, xylem, signals, regulators

## WHY LUPINS AS A MODEL LEGUME?

Although the genome sequence for lupin is not presently available, the genus offers a valuable model for studies that link long distance translocation with plant growth and development. A long history of physiological experiments has linked growth and development of one organ to that of other organs on the plant, leading to the concept that, along with the flow of nutrients and assimilates in the vasculature, regulatory signals are also translocated from sources to sinks. There is good evidence for translocation of a number of the well established plant growth regulators, including auxin, cytokinins (CK), gibberellins, jasmonic acid and precursors for ethylene synthesis (Hoad, 1995).

However, regulatory roles for translocated protein, peptide and nucleic acid molecules are beginning to be described (Atkins and Smith, 2007) and, while many have yet to be identified and their molecular mechanisms of action clarified, powerful roles in gene regulation have been postulated. A classical example is the case of ‘Florigen’, a hypothetical signal translocated from leaves to the shoot apical meristem of a plant to induce flowering in response to specific environmental cues. Its existence was first postulated by the Russian researcher, Chailakhyan (1936), more than 70 years ago. Roles for a number of the known growth regulators have been postulated over the years, and searches of translocation fluids collected from a number of species, including *L. albus*, have identified possible peptide signals (Hoffmann-Behning *et al.* 2002). However, recent data (Huang *et al.* 2005) has implicated mRNA of the *FLOWERING LOCUST (FT)* gene in *Arabidopsis* and/or the FT protein itself (Corbesier and Coupland, 2006) in providing the translocated ‘florigenic’ signal. This latter is supported by the presence of FT in phloem exudate of *B. napus* (Giavalisco *et al.* 2006). With the recent realisation that small RNA species (siRNA and microRNA) are translocated and, potentially at least, influence gene expression at ‘sink’ sites distant to their site of origin, reliable collection and analysis of translocated fluids in plants becomes an essential tool.

In general, collection of the fluid translocated in xylem as a consequence of transpiration is relatively easy. However, in most plants a wound to phloem results in rapid occlusion of the sieve tubes with little or none of their contents exuded. Among the phloem ‘haemophiliacs’ of the plant kingdom are the lupins. Two of the lupin species, *L. angustifolius* and *L. albus*, are amenable to collection of both xylem and phloem exudates. *L. mutabilis* will bleed from wounded vasculature and, under some conditions, this is also true of *L. cosentinii*; but *L. luteus* does not ‘bleed’. Most importantly, phloem exudates can be readily collected at both sources and sinks, permitting construction of quantitative models for assimilate flow (Pate *et al.* 1998) and providing a basis to study molecular events that signal regulation of partitioning. Members of the genus can be grafted and stably transformed (Pigeaire *et al.* 1997). Transformation frequencies for *L. albus*, which bleeds phloem most consistently, are low, but the root system can be transformed using

*Agrobacterium rhizogenes* to generate 'hairy roots' (Uhde-Stone *et al.* 2005). To further develop these species as models for translocation, more molecular information would be useful, particularly expressed sequence tag (EST) libraries and BAC clones, which apart from providing information about the transcriptome, would facilitate further proteomic studies, and development of TILLING (Targeting Induced Local Lesions IN Genomes) systems to identify mutants in key genes (McCallum *et al.* 2000). Information on translocation gained from studying this genus could serve as a model for other pulse (grain legume) crops, most of which suffer from relatively low harvest indices.

### WHAT POTENTIAL SIGNALS ARE FOUND IN PHLOEM EXUDATE FROM LUPINS?

The most abundant solute in phloem exudate is sucrose. While it is the single most important translocated product of photosynthesis, sucrose has also been postulated to serve a signaling role (Rolland *et al.* 2006) in assimilate partitioning and seed development (Weber *et al.* 2005). Prominent phloem-borne amino acids like glutamine and less abundant ones like gamma-aminobutyric acid (GABA) have also been shown to influence the activity of ion channels (Beuve *et al.* 2004). Both are readily recovered in lupin phloem exudates (Atkins *et al.* 1983).

The common plant growth regulators are also found in lupin phloem exudates. Despite a general belief that the prominent translocated forms of CK are the nucleosides detailed analysis of exudates from *L. albus* indicates that there are 12 individual CK species (Emery *et al.* 2000). Significant shifts from *cis* to *trans* isomers in both xylem and phloem appear to be related to changes in reproductive development. No doubt similar studies would reveal changes in the forms and amounts of all the other growth regulators in phloem, but, like the CK, relating such changes to sink organ development and to the specific molecular events that depend on these translocated signals remains obscure.

Recent estimates of the phloem proteome in angiosperms indicate 1500 species (Lough and Lucas, 2006). Mature sieve tubes are enucleate and are thought to be totally dependant on their plasmodesmatal connection to companion cells for the protein products of transcription. In general size exclusion limits appear to be 27-36 kDa but the degree to which these apply at all plasmodesmatal sites in the plant and how they might be regulated is not known. Although a number of studies using tagged antibodies (Terce-Laforgue *et al.* 2004) or GFP-linked expression of gene promoters in transformed plants (Stadtler *et al.* 2005) have localised expression to phloem, they do not prove that the proteins are in fact translocated.

Transcriptomic analysis of lupin phloem exudate reveals a small group of transcripts that could also have signaling functions either in the sieve tube/companion

cell complex or in sink organs as a consequence of translocation (Rodriguez, C., Atkins, C.A. and Smith, P.M.C., unpublished results). There are a number of examples of mRNAs that are translocated in phloem of other species and some where translocation has been demonstrated to result in changes in plant development (Banerjee *et al.* 2006).

Small non-coding RNAs play an important role in gene regulation in eukaryotes. Two classes of small RNA, small interfering RNAs (siRNAs) and microRNAs (miRNAs) have been detected in phloem exudate (Yoo *et al.* 2004; Smith, P.M.C. and Atkins, C.A., unpublished data). These small RNAs interact with a multicomponent RNA-induced silencing complex (RISC) and direct the cleavage of their target RNAs. siRNAs, which were initially identified through their role in co-suppression of transgenes, form a part of the plant's defense mechanism against foreign viruses. They act to silence viral RNAs and so restrict spread of the virus. Once production is initiated a systemic signal is generated to allow a response in distant tissues. Silencing of transgenes in RNAi is also mediated by siRNAs. miRNAs are important regulators of plant development and responses to environmental signals. The majority of their target genes are transcription factors and they play an important role in clearing regulatory transcripts from daughter cell lineages to allow a change in developmental state (Rhoades *et al.* 2002). We have identified eleven different miRNAs in *L. albus* phloem (Jordan M, Atkins CA and Smith PMC, unpublished results and Jordan, 2004). Importantly the spectrum of miRNAs varies with different phloem collection sites on the plant (fruits, upper stem branches and lower main stem) indicating specificity in their synthesis and or loading onto the translocation stream.

### A NOTE ON SAPS/EXUDATES AND THEIR COLLECTION

While the technique of phloem bleeding in lupin sounds attractive there are some precautions that should be considered. The major solutes in phloem, such as sugars, amino acids and other nutrients, are present in exudates in relatively large amounts; for example in *L. albus* sucrose constitutes 20-30% by weight and the single amino acid, asparagine, 1-2% (Layzell *et al.* 1981), and so contamination from a wound is not likely to be significant. However, this is not the case for the cytokinins that occur at levels of 1-50 pmol.ml<sup>-1</sup> (Emery *et al.* 2000) and collections of phloem exudate from *L. albus* require volumes of 5-10 ml or more for transcriptomic and proteomic analysis, particularly for the detection and sequencing of small RNA species (microRNA) (Jordan, 2004). Thus, the source of these 'trace' constituents is as critical as their analysis in determining the significance of their appearance in exudates and their likely roles in signaling or regulating gene expression.

The possibility that molecules in very small amounts found in phloem exudate collected from wounds to the vasculature could be due to contamination from surrounding cells, or artifacts caused by a local 'wound response', must be considered before a role, dependant on their translocation, can be assigned. The most precise method for phloem collection has been considered to be exudate from the detached stylets of sap sucking insects, such as aphids or brown leaf hoppers. While 'stylet exudate' may be regarded as ideal and least subject to artifact, stylets can be extremely variable in both rate and extent of exudation, and, even though the insect body is removed, salivary secretions are likely to persist as contaminants.

Piercing a sieve tube would cause a rapid fall in turgor pressure, decreasing local water potential resulting in both longitudinal and lateral fluxes of water, diluting the exudate and potentially increasing solute fluxes from closely associated companion cells, phloem parenchyma and the tissue apoplast. Structural components of sieve tubes, which are not normally translocated, might also be released by the change in pressure on piercing. For these reasons the first drop of exudate from a vascular incision is typically discarded, ostensibly to minimise contamination. While this precaution may reduce the impact from damaged cells, analyses of solutes from sequentially collected samples of exudate indicate that there is no progressive decline in these despite prolonged 'bleeding' (Eschrich and Heyser, 1975). Estimates based on the assumption that an aphid stylet may enter and draw initially on a single sieve tube, each  $\mu\text{l}$  of exudate is equivalent to the luminal volume of about 2,500 sieve tubes (Dixon, 1975). Thus in lupin, where 50  $\mu\text{l}$  of sap containing ca 10-20% sucrose is readily collected within minutes from an incision in the bundle of vascular elements at the styler tip or along the sutures of a fruit, the exudate could derive from more than  $10^5$  sieve tubes, extending some considerable distance beyond the wound. The volume of contaminating cell contents or apoplast constituents is difficult to estimate but in any case it is likely to be small by comparison. Similar considerations apply to the collection of xylem contents by applying positive or negative pressure to the severed end of stems or the root system. Although the pressures required are relatively gentle, xylem is continuous with the tissue apoplast and constituents such as extracellular proteins for example, which would not be translocated, may be released to the exudate.

While a number of assays have been used to assess the 'purity' of phloem exudate, none is likely to be generally applicable and useful. Determining translocation of any putative signal molecule in phloem exudates needs to be proven directly rather than inferred and their functional significance revealed by demonstrating a translocation-dependant role.

## LITERATURE CITED

- Atkins C.A., J.S. Pate, M. Peoples and K. Joy. 1983. Amino acid transport, and metabolism in relation to the nitrogen economy of a legume leaf. *Plant Physiology* 71: 841.
- Atkins, C.A. and P.M.C. Smith. 2007. Translocation in Legumes: Assimilates, Nutrients and Signaling molecules. *Plant Physiology* 144: 550.
- Banerjee, A.K., M. Chatterjee, Y. Yu, S.G. Suh, W.A. Miller and D.J. Hannapel. 2006. Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. *Plant Cell* 18: 3443.
- Beuve, N., N. Rispail, P. Laine, J-B. Cliquet, A. Ourry and Le E. Deunff. 2004. Putative role of  $\gamma$ -aminobutyric acid (GABA) as a long-distance signal in up-regulation of nitrate uptake in *Brassica napus* L. *Plant Cell and Environment* 27: 1035.
- Chailakhyan, M.Kh.1936. New facts in support of the hormonal theory of plant development. *C. R. Acad. Sci. URSS* 13: 79.
- Corbesier, L. and G. Coupland. 2006 The quest for florigen: a review of recent progress. *J Exp Bot* 57: 3395.
- Dixon, A.F.G. 1975. Aphids and translocation. In eds MH Zimmermann and JA Milburn, eds, *Transport in Plants I Phloem Transport*, Springer Verlag, Berlin, pp. 154-170.
- Emery, R.J.N., Q.F. Ma and C.A. Atkins. 2000 The forms and sources of cytokinins in developing white lupine seeds and fruits. *Plant Physiology* 123: 1593.
- Eschrich, W. and W. Heyser. 1975. Biochemistry of phloem constituents. In MH Zimmermann JA Milburn, eds, *Transport in Plants I Phloem Transport*, Springer Verlag, Berlin, pp. 101-136.
- Giavalisco, P., K. Kapitza, A. Kolasa, A. Buhtz and J. Kehr. 2006. Towards the proteome of *Brassica napus* phloem sap. *Proteomics* 6: 896.
- Hoad, G.V. 1995. Transport of hormones in the phloem of higher plants. *Plant Growth Regulation* 16: 173.
- Hoffmann-Benning, S., D.A. Gage, L. McIntosh, H. Kende and J.A.D. Zeevaart. 2002. Comparison of peptides in the phloem sap of flowering and non-flowering *Perilla* and lupine plants using microbore HPLC followed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Planta* 216: 140.
- Huang, T., H. Böhlenius, S. Eriksson, F. Rarcy and O. Nilsson. 2005. The mRNA of the *Arabidopsis* gene *FT* moves from leaf to shoot apex and induces flowering. *Science* 309: 1694.
- Jordan, M.E. 2004. Micro RNAs in *Lupinus albus* (L.). Honours Thesis, the University of Western Australia.
- Layzell, D.B., J.S. Pate, C.A. Atkins and D.T. Canvin. 1981. Partitioning of carbon and nitrogen and nutrition of root and shoot apex in a nodulated legume. *Plant Physiology* 67: 30.
- Lough, T.J. and W.J. Lucas. 2006. Integrative plant biology: role of phloem long distance macromolecular trafficking. *Annual Review of Plant Biology* 57: 203.
- McCallum, C.M., L. Comai, E.A. Greene and S. Henikoff. 2000. Targeted screening for induced mutations. *Nature Biotechnology* 18: 455.

- Pate, J.S., R.J.N. Emery and C.A. Atkins. 1998. Transport Physiology and Partitioning. *IN*: J.S. Gladstones, C.A. Atkins, J. Hamblin, eds, *Lupins as Crop Plants: Biology, Production and Utilisation*, CAB International, Wallingford, Oxon UK. pp. 181-226.
- Pigeaire, A., D. Abernathy, P.M. Smith, K. Simpson, N. Fletcher, C-Y. Lu, C.A. Atkins and E. Cornish. 1997. Routine transformation of a grain legume crop (*Lupinus angustifolius* L.) via *Agrobacterium tumefaciens*-mediated gene transfer to shoot apices. *Molecular Breeding* 3: 341.
- Rhoades, M.W., B.J. Reinhart, L.P. Lim, C.B. Burge, B. Bartel and D.P. Bartel. 2002. Prediction of plant microRNA targets. *Cell* 110: 513.
- Rolland, F., E. Baena-Gonzalez and J. Sheen. 2006. Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review Plant Biology* 57: 675.
- Stadtler, R., K.M. Wright, C. Lauterbach, G. Amon, M. Gahrtz, A. Feuerstein, K.J. Oparka and N. Sauer. 2005. Expression of GFP-fusions in *Arabidopsis* companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. *Plant Journal* 41: 319.
- Terce-Laforgue, T., F. Dubois, S. Ferrario-Mery, M-A. Pou de Crezenzo, R. Sangwan and B. Hirel. 2004. Glutamate dehydrogenase of tobacco is mainly induced in the cytosol of phloem companion cells when ammonia is provided either externally or released during photorespiration. *Plant Physiology* 136: 4308.
- Uhde-Stone, C., J. Liu, K.E. Zinn, D.L. Allan and C.P. Vance. 2005. Transgenic proteoid roots of white lupin: a vehicle for characterising and silencing root genes involved in adaptation to P stress. *Plant Journal* 44: 840.
- Weber, H., L. Borisjuk and U. Wobus. 2005. Molecular physiology of legume seed development. *Annual Review Plant Biology* 56: 253.
- Xoconostle-Cazares, B., Y. Xiang, R. Ruiz-Medrano, H.L. Wang, J. Monzer, B.C. Yoo, K.C. McFarland, V.R. Franceschi and W.J. Lucas. 1999. Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. *Science* 283: 94.
- Yoo, B.C., F. Kragler, E. Varkonyi-Gasic, V. Haywood, S. Archer-Evans, Y.M. Lee, T.J. Lough and W.J. Lucas. 2004. A systemic small RNA signaling system in plants. *Plant Cell* 16: 1979.