

Unexpected Metabolites in Tobacco Genetically Modified to Accumulate Selenium

Adam J. Matich,^a Nigel I. Joyce,^b Marian J. McKenzie,^a David A. Brummell,^a Daryl D. Rowan.^a

The New Zealand Institute for Plant and Food Research Limited, ^aPrivate Bag 11600, Palmerston North, ^bPrivate Bag 4704, Christchurch (e-mail: daryl.rowan@plantandfood.co.nz)

Selenium Accumulation by Plants

Most plants cannot tolerate high levels of selenium in the soil as the enzymes of the sulfur assimilation pathway do not distinguish between sulfur and selenium and consequently both are taken up by the plant.¹ Inorganic selenium is metabolised to selenocysteine and selenomethionine, the selenium analogues of cysteine and methionine, which are then incorporated into plant proteins causing defective secondary structure and reduced enzymatic activity, observed as stunting, necrotic lesions on the leaves, and reduced root growth.² Selenium non-accumulators are plants that cannot tolerate high-selenium soils, although in low-selenium soils they can discharge some selenium as dimethylselenide (MeSeMe).^{1,2} In contrast, selenium accumulators such as garlic (*Allium sativum*) and *Brassica* species, e.g. broccoli, and hyperaccumulators, such as the two-grooved milk vetch (*Astragalus bisulcatus*), will tolerate high-selenium soils because they metabolise absorbed selenium so as to remove it from the pool of substrates that can be incorporated into proteins. The two-grooved milk vetch can accumulate several grams of selenium per kilogram of dry matter without showing signs of toxicity.

Selenium-accumulators use a selenocysteine methyltransferase (SMT) enzyme to remove selenocysteine from the cysteine pool by methylating it to methylselenocysteine (MeSeCys).^{1,2} In young leaves of the two-grooved milk vetch, most of the selenium accumulates as MeSeCys, although some is transformed into γ -glutamyl-MeSeCys, or volatilized as dimethyl diselenide (MeSe₂Me).^{2,3} The organoselenides produced by different types of plants are characteristic of their selenium accumulation status. Non-accumulators produce only MeSeMe, whereas in the two-grooved milk vetch MeSe₂Me is the major volatile. Only in selenium accumulators, where selenium flow is diverted into MeSeCys, can MeSe₂Me be produced, and its generation is therefore indicative of the presence of SMT activity. The ability to synthesise MeSeCys gives such plants an additional capacity to discharge a proportion of the accumulated selenium as volatiles.

The modification of selenium biochemistry in plants is of interest because of the essential role of selenium in human nutrition and health and the possible application of selenium volatilisation to the phytoremediation of selenium contaminated soils. Selenium is an essential micronutrient and may also play a role in cancer prevention as evidenced by the anti-carcinogenic activity of MeSeCys against animal cancer cell lines.^{4,5} Although supplementation with selenium may help reduce the risk of cancer, the form in which the selenium is ingested is important. Rat cancer models fed with high-selenium broccoli showed an additional protection against cancer, when compared with control rats fed equivalent amounts of inorganic selenium

and regular broccoli.^{6,7} This is believed to be because these plants accumulate MeSeCys which is thought to have anti-cancer activity when converted to methylselenol in mammals.^{8,9} Selenium-accumulators can also be used for phytoremediation of high-selenium soils,^{10,11} in which case volatilisation of selenium may be more desirable than its storage, as selenium is dispersed from the local area as volatile compounds that are less toxic than those found in the soil.^{10,12} This enables phytoremediation plantings to have a longer useful life, avoiding the need for harvesting and replanting to remove the accumulated selenium. Selenium volatilization from non-accumulators is enhanced by the expression of *SMT* or cystathione- γ -synthase transgenes in species used for phytoremediation.¹² Although MeSe₂Me is the major organoselenium volatile produced by engineered selenium accumulators, MeSeMe is also produced in large quantities.^{13,14}

A by-product of the genetic modification of plants may be the biosynthesis of new compounds, the discovery of which is aided by technological improvements to our ability to detect and measure multiple metabolites in complex biological samples. Re-direction of selenium metabolism and characterisation of the resulting compounds may shed light on presently unknown mechanisms and pathways for selenium assimilation in plants. We illustrate the process of metabolite identification using some new organoselenide semi-volatiles detected in tobacco genetically modified to accumulate selenium.

Organoselenides in Transgenic Tobacco Leaves

Two populations of transgenic tobacco plants were created: one constitutively over expressing an *Astragalus bisulcatus* *SMT* transgene, and the other constitutively over expressing both the *SMT* transgene and a broccoli *ATP sulfurylase* gene.¹⁴

LC-MS analysis with selective reaction monitoring (SRM) was used to selectively measure the selenium containing amino acids, MeSeCys and γ -glutamyl-MeSeCys, in the leaves after watering of the plants with sodium selenate for a period of 14 days (Fig. 1). Use of SRM allowed the sensitive quantitation of these amino acids by detection of specific fragment ions produced in the ion trap. The absolute concentration of γ -glutamyl-MeSeCys could not be determined owing to the absence of an authentic standard, but relative amounts of this metabolite were readily measured. In wild-type controls, neither MeSeCys nor MeCys was present much above the detection limit of 0.1 ng on column, but all transgenic lines over expressing *SMT* accumulated substantial amounts of MeSeCys: up to 5% of the plant's total accumulated selenium. MeSeCys accumulated to a greater extent in selenate-watered plants transformed

with both the *ATPS* and *SMT* transgenes. Concentrations ranged from 0.89 to 1.47 g/kg DW and constituted up to 10% of the plant's total accumulated selenium at concentrations around 10-fold higher than that of MeCys.¹⁴

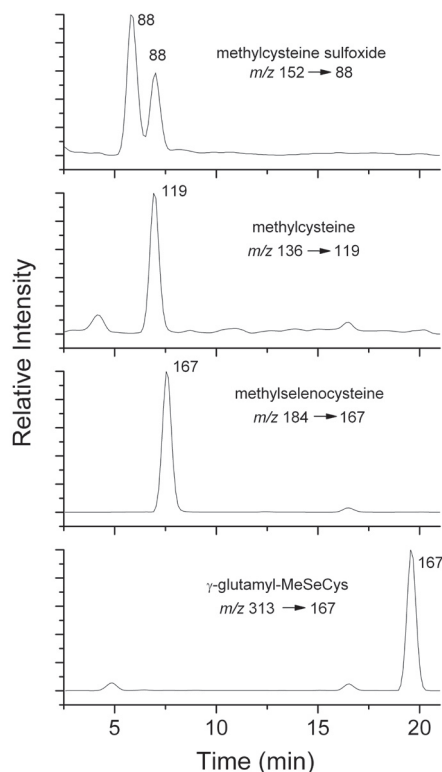


Fig. 1. Analysis of sulfur and selenium amino acids in transgenic tobacco leaf by ion trap LC-MS showing selected ion chromatograms of fragment ions selected for quantitation by selective reaction monitoring (SRM).

Organoselenides in the Headspace above Transgenic Tobacco Plants

Watering of transgenic plants with sodium selenate resulted in a distinctive off-odour of *cabbage* or *ocean* in the glasshouse, suggesting the production of volatile organoselenides. To identify these compounds, individual tobacco plants were enclosed in oven bags and sealed about the stem just above the level of the potting mix. The headspace in the bag was allowed to equilibrate for one hour prior to sampling with a CarboxenTM-PDMS solid phase microextraction (SPME) fibre inserted through the wall of the oven bag and maintained in position overnight. Volatiles were then desorbed from the SPME fibres in the injection port of the gas chromatograph. GC-MS analysis showed the presence of MeSeMe, MeSeSMe, and MeSe₂Me.¹⁴ These organoselenides were located in the GC-MS traces by their distinctive isotopic patterns and the mass deficiency of selenium-containing ions, and by using distinctive fragment ions to generate selective ion chromatograms (Fig. 2). The most useful of these fragment ions was *m/z* 92.9 (CH⁸⁰Se⁺) found in all of the organoselenides.

The formation of MeSeMe from selenomethionine does not require SMT activity and so this compound was found in both the engineered and the wild-type plants. MeSeSMe and MeSe₂Me were found only in the volatiles collected from transgenic plants fertilized with sodium selenate.¹⁴ All transgenic plants produced substantial amounts of

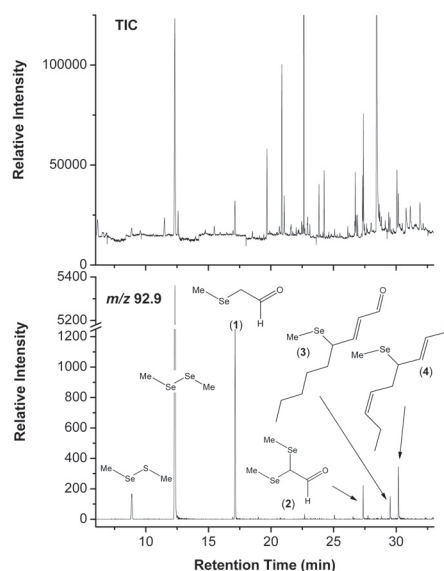


Fig. 2. Simplification of the complex metabolic profile of tobacco leaf extracts and detection of organoselenides achieved using *m/z* 92.9.

MeSe₂Me, which is formed from MeSeCys, indirectly confirming the presence of SMT activity in these plants. MeSe₂Me is the volatile organoselenide compound most commonly reported in selenium accumulating organisms^{13,15,16} and was found in the headspace at similar concentrations to MeSeMe. MeSeMe and MeSe₂Me are commercially available, but MeSeSMe is not and so was identified by high resolution (accurate mass) EI-GCMS. Thus, MeSeSMe showed a molecular ion at *m/z* 141.9360 (C₂H₆SeS⁺) and was identified by fragmentation analysis and comparison with the literature.¹⁷ MeSeSMe may arise by disproportionation of MeSe₂Me with MeS₂Me, traces of which were detected in some plants.

Semi-volatile Organoselenides in Leaf Extracts of Transgenic Tobacco Plants

Four new organoselenides (**1-4**, Chart 1) were identified in crude solvent extracts from the leaves of the transgenic tobacco plants (Fig. 2), based on the very distinctive isotope pattern and mass deficiency of selenium, and the seemingly ubiquitous fragment cluster at around *m/z* 92.9. As insufficient sample was available for isolation and NMR analysis, possible structures were deduced from the mass spectral data and candidate compounds were prepared by synthesis for GC-MS comparison with the compounds in the plant extracts.¹⁸

The first new metabolite, 2-(methylseleno)acetaldehyde (**1**, Chart 1) was the second most prevalent organoselenide in the solvent extracts (17.3 min, Fig. 2). The molecular ion for C₃H₆OSe was at *m/z* 137.9586, (137.9584 required), and a cluster of ions centred around *m/z* 108.9554 (C₂H₅Se⁺, M⁺-CHO) suggested that **1** was an aldehyde. Methylselenoacetate is one candidate compound, but the expected large base peak at *m/z* 43 was not observed (Fig. 3).¹⁹ Aldehyde **1** seemed the most likely structure and the mass spectrum bore some similarity to that reported previously.²⁰ Synthesis of this compound confirmed that its GC retention time and mass spectrum (Fig. 3) matched that of the tobacco metabolite.

Chart 1. Orgaoselenides 1-4 identified in leaves from transgenic tobacco plants, and synthetic compounds 5-7

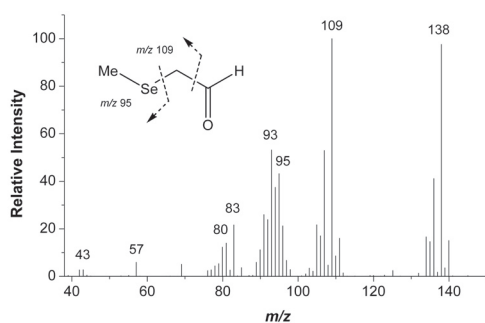
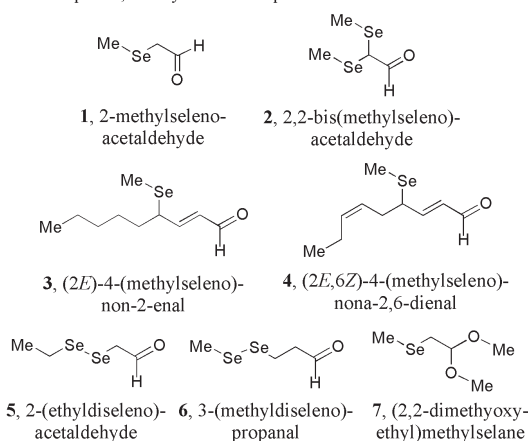
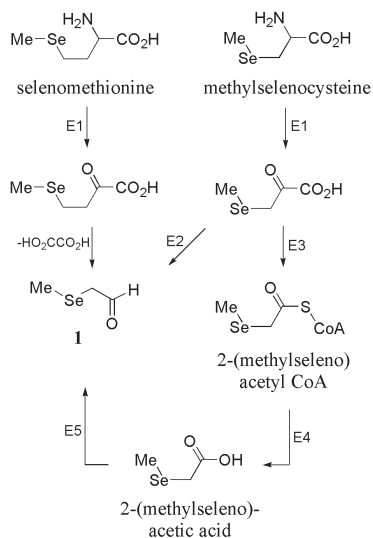


Fig. 3. Mass spectrum of 2-(methylseleno)acetaldehyde (**1**) found in leaves of transgenic tobacco plants treated with Na_2SeO_3 (redrawn from Matich *et al.* - see ref. 18).

Determination of the structure of **1** identifies a new pathway of selenium metabolism in tobacco that diverges from those responsible for the production of the well known organoselenides, MeSeMe and MeSeSeMe.¹⁴ Aldehyde **1** may derive from either selenomethionine or MeSeCys, by mechanisms similar to those observed for the catabolism of branched-chain amino acids in bacteria and yeast, as shown in Scheme 1.



Scheme 1. Possible biosynthetic pathways for 2-(methylseleno)acetaldehyde (**1**) in transgenic tobacco leaves from selenomethionine (ref. 21) and MeSeCys. E1: amino acid amino transferase, E2: 2-oxoacid decarboxylase, *c.f.* pyruvate decarboxylase, E3: 2-oxoacid dehydrogenase, E4: acyl-CoA hydrolase (see ref. 22), E5, acyl-CoA reductases see (ref. 23).

The second new organoselenide **2** (retention time of 27.3 min, Fig. 2) contained two selenium atoms, as shown by the isotopic distribution (Fig. 4) and the high resolution mass spectrum of its molecular ion $\text{C}_4\text{H}_8\text{OSe}_2$ (m/z 231.8935). The fragment ions $\text{C}_3\text{H}_7\text{Se}_2^+$ (m/z 202.8878) and CH_3Se_2^+ (m/z 175) indicated M^+-CHO and of $\text{M}^+-\text{C}_2\text{H}_4\text{CHO}$, respectively, suggesting this metabolite was another aldehyde but also containing a Se-Se bond. However, the absence of m/z 160 (Se_2^+) put this in dispute. The fragment ion m/z 109 ($\text{C}_2\text{H}_5\text{Se}^+$) corresponds to $\text{M}^+-\text{SeCH}_2\text{CHO}$ implying that **2** contains an ethyl group. The candidate compounds 2-(ethyldiseleno)acetaldehyde (**5**) and 3-(methyldiseleno)propanal (**6**) were synthesised, but their mass spectra did not match that of tobacco compound **2**. Most notably, the mass spectra of these compounds contained m/z 43 (CH_2CHO^+) and m/z 57 ($\text{CH}_2\text{CH}_2\text{CHO}^+$), respectively, and in particular the Se-Se⁺ moiety (m/z 160), which were not present in **2** (Fig. 4).

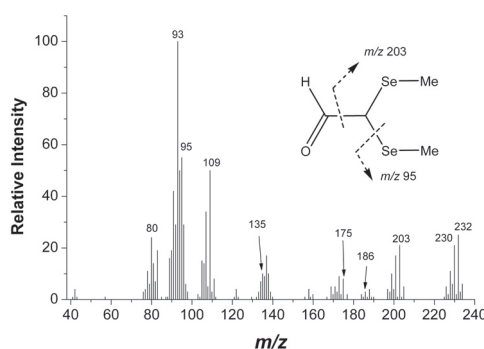
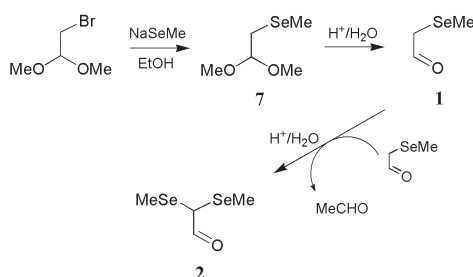


Fig. 4. Mass spectrum of 2,2-bis(methylseleno)acetaldehyde (**2**) found in leaves of transgenic tobacco plants treated with Na_2SeO_3 (redrawn from Matich *et al.* - see ref. 18).

Fortunately, we noticed that a by-product from the synthesis of aldehyde **1** (Scheme 2) had the same GC retention time and mass spectrum as **2** and to 2,2-bis(methylseleno)acetaldehyde **2**, a by-product previously reported from acid hydrolysis of the ethyl acetal analogue of **7**.²⁰ Therefore, the above mentioned ion at m/z 175 (MeSe_2^+) did not arise from a Se-Se bond and probably results from rearrangement of $(\text{MeSe})_2\text{CH}^+$ (m/z 203) to $\text{MeSeSe}^+\text{CHMe}$ prior to fragmentation. *In planta*, aldehyde **2** would also seem to arise from acid-catalysed disproportionation of aldehyde **1** (Scheme 2).



Scheme 2. Synthesis of 2-(methylseleno)acetaldehyde (**1**) and its acid-catalysed conversion into diselenide **2** during hydrolysis of acetal **7**.

The third and fourth organoselenides **3** ($\text{C}_{10}\text{H}_{18}\text{OSe}$) and **4** ($\text{C}_{10}\text{H}_{16}\text{OSe}$) were found at their highest levels in leaves from doubly transformed plants. Their mass spectral fragmentation patterns below m/z 110 suggested these compounds differed by one double bond. In aldehyde **4**, m/z 163 ($\text{C}_5\text{H}_7\text{SeO}^+$) corresponded to $\text{M}^+-\text{C}_5\text{H}_9$ and m/z 135

($C_4H_7Se^+$) to the subsequent loss of CO (Fig. 5). Some of the ions clustered around m/z 135 also represented the loss of SeMe and MeSeH (m/z 137 and 136, respectively), to give a nine-carbon fragment. These fragmentations suggest that aldehyde **4** might have a 4-(methylseleno)-2,6-nonadienal structure. (2*E*,6*Z*)-4-(Methylseleno)nona-2,6-dienal was synthesised by adding the nonadienal to (2,2-dimethoxyethyl)(methyl)selane (**7**) prior to its acid hydrolysis, with the expectation that during the hydrolysis a reaction similar to that shown in Scheme 2 for the production of (MeSe)₂CH₂CHO (**2**) would occur. Selective TOCSY NMR experiments confirmed that aldehyde **4** was in fact produced, and the mass spectrum (Fig. 5) and retention time of this synthetic compound matched to those of the tobacco compound. Aldehyde **3** was similarly synthesised from (2*E*)-nonenal and (**7**) and characterised as (2*E*)-4-(methylseleno)non-2-enal. Given the reactivity of aldehyde **1** with (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal, under acid conditions *in vitro*, aldehydes **2-4** may well result from spontaneous chemical reaction in the plants or in the solvent extracts. Regardless, the presence of these compounds demonstrates the reactivity and potential of **1** to participate in interesting chemistry *in vivo*.

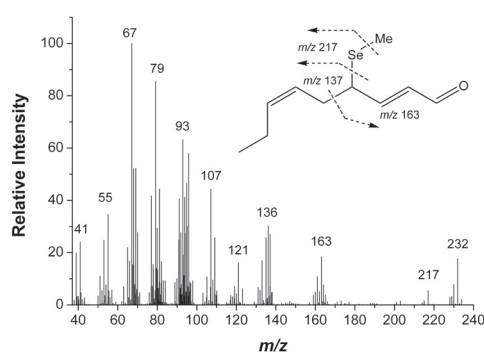


Fig. 5. Mass spectrum of (2*E*,6*Z*)-4-(methylseleno)nona-2,6-dienal (**4**) found in leaves of transgenic tobacco plants treated with Na₂SeO₃ (redrawn from Matich *et al.* – see ref. 18).

We have shown that a Solanaceous species (tobacco), lacking the sulfur secondary metabolism found in the *Brassicaceae*, can be converted from a selenium non-accumulator into a selenium accumulator by genetic modification. This work extends previous studies in *Arabidopsis* and Indian mustard and demonstrates that the trait of MeSeCys accumulation can be moved from the Se-hyperaccumulators to plants outside of the *Brassicaceae*. Transformation of tobacco into an accumulator of MeSeCys¹⁴ resulted in the production of additional organoselenide metabolites, in particular aldehyde **1**, whose presence suggests the operation of a new pathway for selenium mobilisation in plants. The other new metabolites may arise by chemical reactions occurring *in planta* or in the plant extracts, but their identification demonstrates the usefulness of GC-MS metabolic profiling in assessing the chemical composition of genetically modified organisms.

Acknowledgements

We thank Ian King for care of the plants, Martin Hunt for GC-MS and Pat Edwards for NMR. This work was funded by the NZ Foundation for Research, Science and Technology contract CO6X0207 and by Vital Vegetables®, a research project jointly funded by Horticulture Australia Ltd., Plant and Food Research, FRST, the Victorian Department of Primary Industries, the NZ Vegetable and Potato Growers Federation Inc. and the Australian Vegetable and Potato Growers Federation Inc.

References

- Terry, N.; Zayed, A. M.; de Souza, M. P.; Tarun, A. S. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **2000**, *51*, 401-432.
- Sors, T. G.; Ellis, D. R.; Salt, D. E. *Photosynth. Res.* **2005**, *86*, 373-389.
- Freeman, J. L.; Zhang, L. H.; Marcus, M. A.; Fakra, S.; McGrath, S. P.; Pilon-Smits, E. A. H. *Plant Physiol* **2006**, *142*, 124-134.
- Kim, T.; Jung, U.; Cho, D. Y.; Chung, A. S. *Carcinogenesis* **2001**, *22*, 559-565.
- Whanger, P. D. *Brit. J. Nutr.* **2004**, *91*, 11-28.
- Finley, J. W.; Davis, C. D. *Biofactors* **2001**, *14*, 191-196.
- Finley, J. W.; Davis, C. D.; Feng, Y. J. *Nut.* **2000**, *130*, 2384-2389.
- Ip, C.; Thompson, H. J.; Zhu, Z. J.; Ganther, H. E. *Cancer Res.* **2000**, *60*, 2882-2886.
- Finley, J. W. *J. Sci. Food Agric.* **2007**, *87*, 1620-1629.
- de Souza, M. P.; Pilon-Smits, E. A. H.; Lytle, M.; Hwang, S., *et al.* *Plant Physiol.* **1998**, *117*, 1487-1494.
- de Souza, M. P.; Pickering, I. J.; Walla, M.; Terry, N. *Plant Physiol.* **2002**, *128*, 625-633.
- LeDuc, D. L.; Tarun, A. S.; Montes-Bayon, M.; Meija, J., *et al.* *Plant Physiol.* **2004**, *135*, 377-383.
- Kubachka, K. M.; Meija, J.; LeDuc, D. L.; Terry, N.; Caruso, J. *Environ. Sci. Technol.* **2007**, *41*, 1863-1869.
- McKenzie, M. J.; Hunter, D. A.; Pathirana, R.; Watson, L. M., *et al.* *Transgenic Res.* **2009**, *18*, 407-424.
- Meija, J.; Bryson, J. M.; Vonderheide, A. P.; Montes-Bayon, M.; Caruso, J. A. *J. Agric. Food Chem.* **2003**, *51*, 5116-5122.
- Shah, M.; Meija, J.; Caruso, J. A. *Anal. Chem.* **2007**, *79*, 846-853.
- Chasteen, T. G. *Appl. Organomet. Chem.* **1993**, *7*, 335-342.
- Matich, A. J.; McKenzie, M. J.; Brummell, D. A.; Rowan, D. D. *Phytochemistry* **2009**, *70*, 1098-1106.
- Tsai, J. H.; Hiserodt, R. D.; Ho, C.-T.; Hartman, T. G.; Rosen, R. T. *J. Agric. Food Chem.* **1998**, *46*, 2541-2545.
- Adlington, R. M.; Barrett, A. G. M. *J. Chem. Soc. Perkin. Trans. 1*, **1981**, *11*, 2848-2863.
- Bonnaime, P.; Amarita, F.; Chambellon, E.; Semon, E., *et al.* *FEMS Microbiol. Lett.* **2004**, *236*, 85-90.
- Dickinson, J. R.; Harrison, S. J.; Dickinson, J. A.; Hewlins, M. J. E. *J. Biol. Chem.* **2000**, *275*, 10937-10942.
- Perpète, P.; Duthoit, O.; De Maeyer, S.; Imray, L.; *et al.* *FEMS Yeast Res.* **2006**, *6*, 48-56.