# New Plant Growth Regulators and an Update on Established Plant Growth Regulators

*Phyto*Technology Laboratories
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SIVB, St. Louis, MO

# Outline

- Grouping of Plant Growth Hormones
- New Plant Growth Regulators/Hormones
  - Brassinosteroids
  - Peptides
  - Strigolactones
  - Melatonin
- Stability of Cytokinins/Auxins
  - Adenine-based cytokinins
  - Thidiazuron
  - Indole butyric acid & Indole acetic acid

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# 5 Groupings of Plant Hormones

The Plant Cell, Vol. 9, 1197–1210, July 1997

## The Five "Classical" Plant Hormones

**Hans Kende and Jan A. D. Zeevaart**[1]

Michigan State University–Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824-1312

- Cytokinins
- Auxins
- Ethylene
- ABA
- Gibberellins

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# New Groupings of Plant Growth Hormones

1. Cytokinins
2. Auxins
3. Stress (Ethylene, ABA, JA, SA)
4. Gibberellins (GA) and Brassinosteroids (BR)
5. Peptides
6. Strigolactones

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# Hormones v. PGR's

- "Plant hormones are *a group of naturally occurring, organic substances which influence physiological processes at low concentrations*." (Davies 2010)

- Hormones
  - Cytokinins (t-Z, 2iP)
  - Auxins (IBA, IAA)
  - ABA
  - GA & Brassinolides
  - Ethylene
  - Strigolactones
  - Plant Defense molecules (Salicylic Acid & Jasmonates)
  - Peptides

- Synthetic
  - BA
  - NAA
  - 2,4-d
  - TDZ
  - 24-epibrassinolide
  - GR24

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# Similar Effects of Gibberellins & Brassinosteroids

- Regulate seed germination
- Increase cell elongation

Deficiency can result in:

- Dwarfism
- Male Sterility

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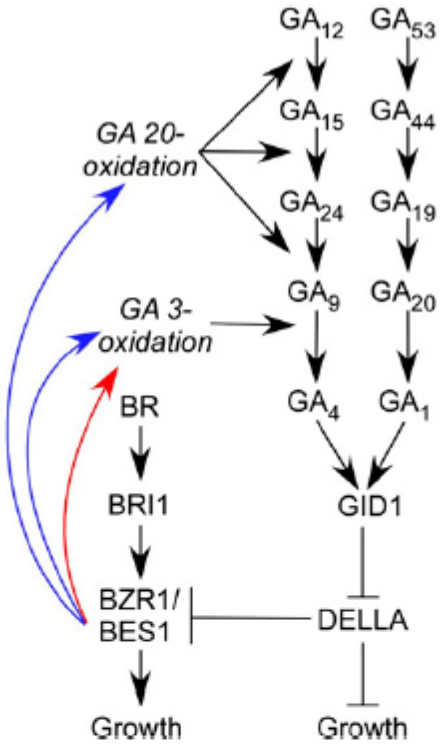
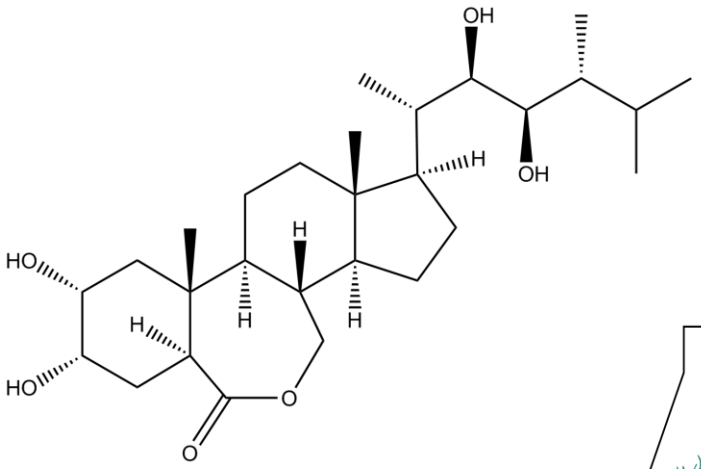
# Brassinosteroids (BR) regulate Gibberellins (GA)

- It has been recently shown BR influence GA biosynthesis and metabolism (Unterholzner *et al.* 2015)
- 24-Epibrassinolide (Prod. No. E244) is the most-widely used synthetic analogue




Ross & Quittenden 2016




24-Epibrassinolide

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# Working with Peptides

- Peptides and proteins can irreversibly bind to the surfaces of plastic and glass tubes and bottles at concentrations near or below 10 μg/mL
- Obviously this is peptide sequence dependent. If your peptide has a lot of β-sheet character it can be worse than perhaps a peptide with a high charge
- To overcome this we would recommend dilutions below 1.0 mg/mL be performed with an aqueous solution of 0.05M NaCl (S624) and 0.1 mg/mL protein
  - Casein (C184)
  - Bovine serum albumin (BSA)

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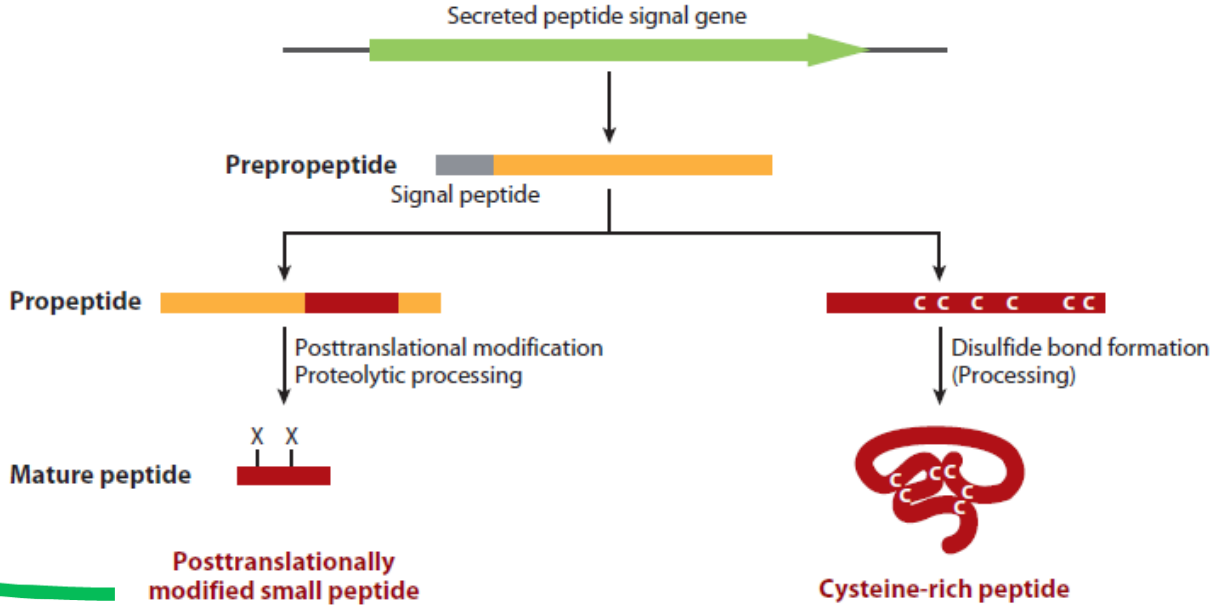
# Peptides

Leucine-rich repeat receptors




- Chains of amino acids linked
- Peptide typically 10 amino acids or less
- Oligopeptide 10-20 amino acids
- Polypeptide 10-40 amino acids
- Proteins >40 amino acids

| Posttranslationally modified small peptides | Cysteine-rich peptides |
|---|---|
| PSK | LAT52 |
| CLV3 | SCR/SP11 |
| CLE | RALF |
| HypSys | TPD1 |
| IDA | EA1 |
| TDIF | EPF |
| PSY | LURE |
| CEP | STOMAGEN |
| CLE-RS | EC1 |
| RGF | |

Matsubayashi 2014

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# Peptide Diversity within Plants

- Peptides generally from nonfunctional precursors
  - Some peptides originate from functional proteins
- Cell-to-Cell signaling is essential to plant growth and development

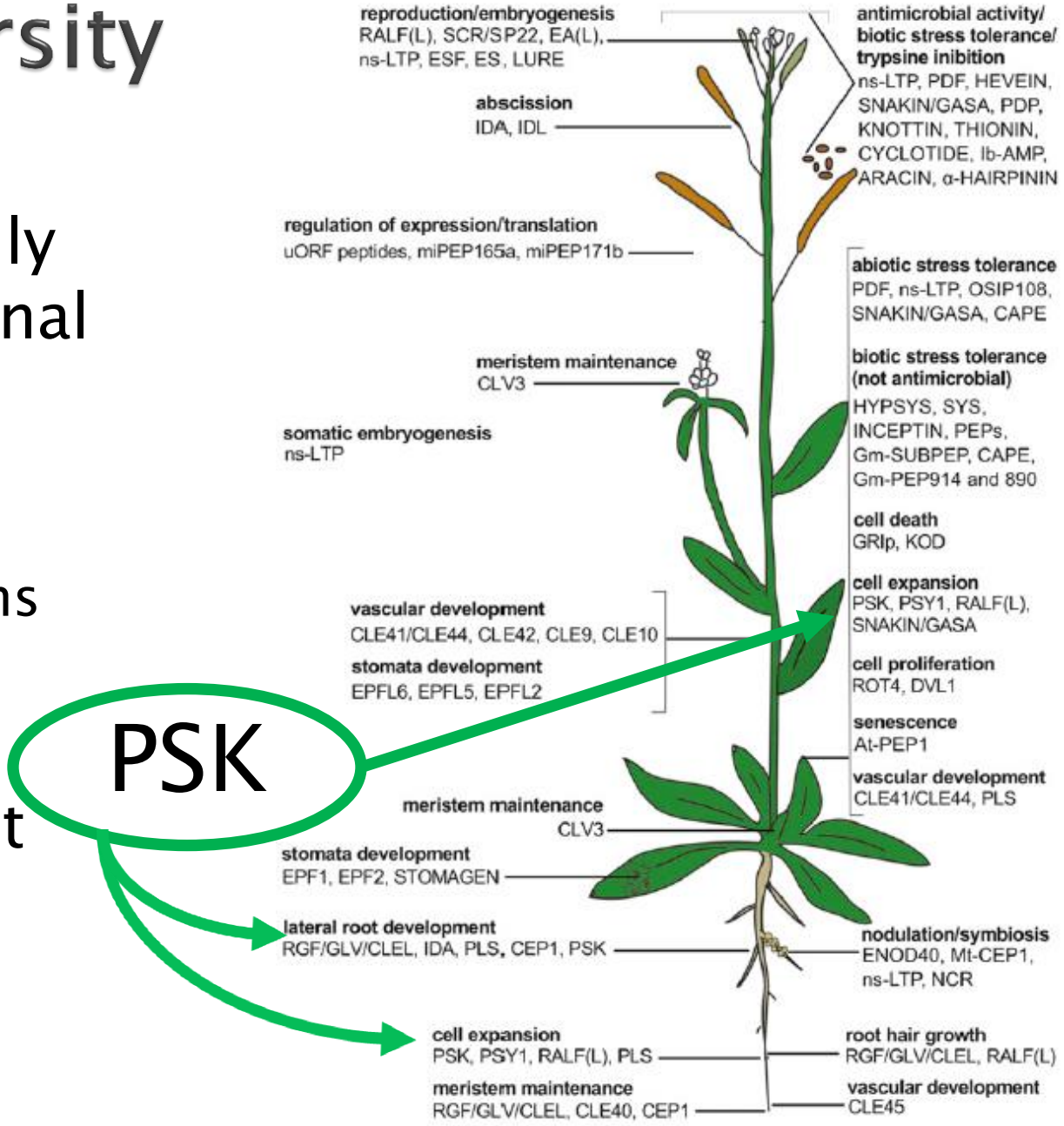
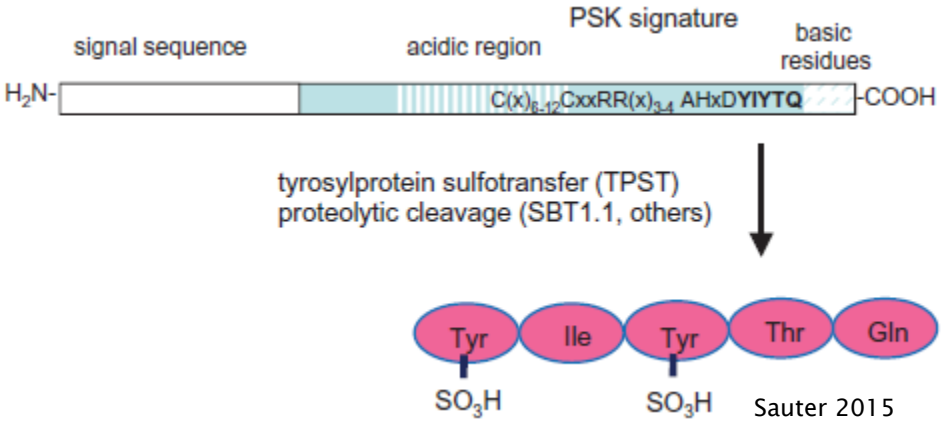



**Figure 2.** The Functional Diversity of Plant Peptides.

Tavormina *et al.* 2015

# PSK-α (Prod. No.P6633)

- Phytosulfokine-alpha (PSK-α)
- First isolated from conditioned medium of mesophyll asparagus cell (Matsubayashi & Sakagami 1996)
  - Present in both monocots and dicots
- Tyrosine-Sulfated pentapeptide with sequence YIYTQ which is heat-stable




Sauter 2015

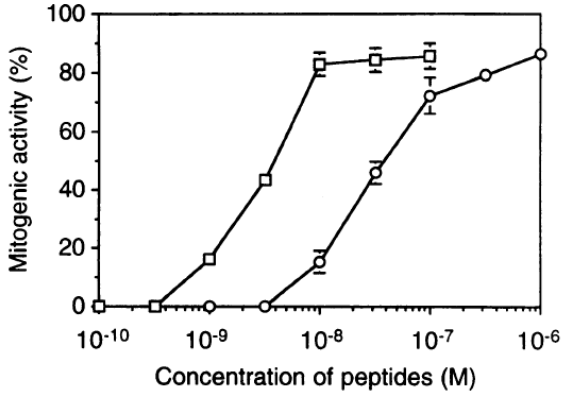



FIG. 6. Mitogenic activities of the natural PSK-α (open squares) and PSK-β (open circles). Single cells of asparagus were cultured at a cell density of $4.0 \times 10^4$ cells/ml in the liquid medium containing various concentrations of natural PSK-α and PSK-β.

Matsubayashi & Sakagami 1996

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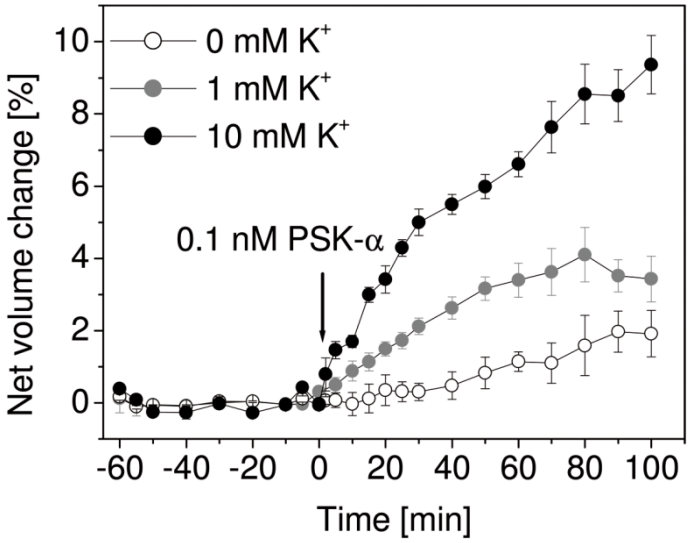
# PSK-α (cont.)

- Induces proliferation of low-density suspension cell cultures
- Enhances lateral root formation (Kutschmar *et al.* 2009)
- Promote primary root growth through cell size increase in the elongation-differentiation zone (Oh *et al.* 2018)
- Increases hypocotyl length by signaling osmotically-driven cell expansion (Stührwohldt *et al.* 2011)
- Enhances microspore embryogenesis in triticale and wheat (Asif *et al.* 2014)


Kutschmar *et al.* 2009




Stührwohldt *et al.* 2011

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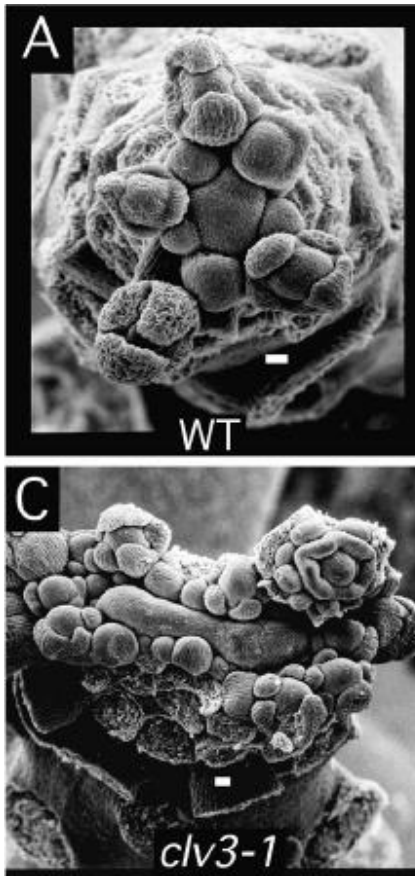
# CLV3 peptide

- CLV3 was discovered first as a regulator of shoot & floral meristem development (Clark *et al.* 1995)
  - Restricts the pluripotent stem cell population in meristems
  - Loss of CLV3 results in enlarged apical meristem
  - Overexpression can lead to decreased stem cell population
- Its true application is tied in with WUSCHEL in a feedback loop (Schoof *et al.* 2000)
- The prolines must be hydroxylated and then arabinosylated for full activity (Ohyama *et al.* 2009)


Czyzewicz *et al.* 2015




Clark *et al.* 1995

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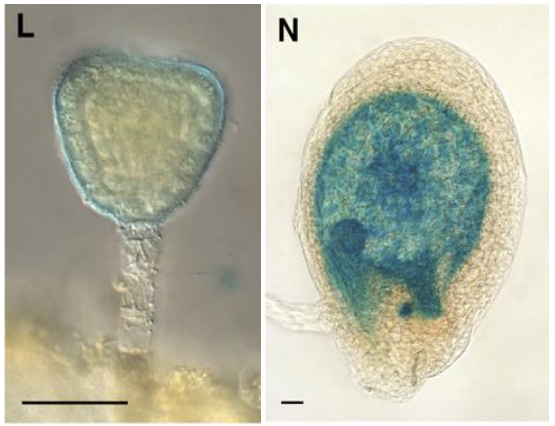
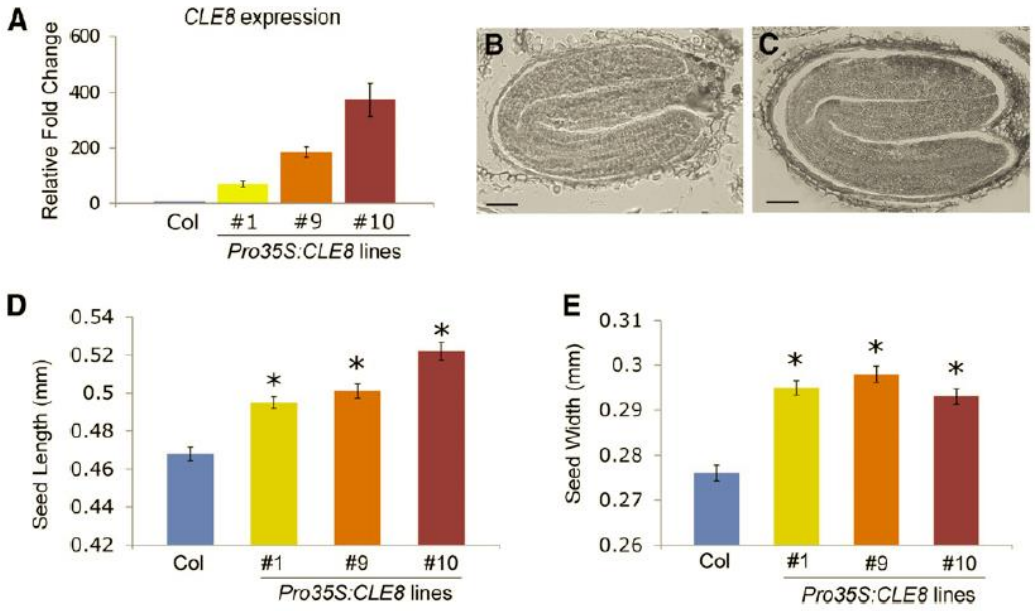
# CLE8 peptide

- CLAVATA3/EMBRYO SURROUNDING REGION-RELATED8 (CLE8)
- Critical for embryo and endosperm development (Fiume & Fletcher 2012)
- Regulates expression of the transcription factor WOX8
  - This increases seed growth and size


Czyzewicz *et al.* 2015

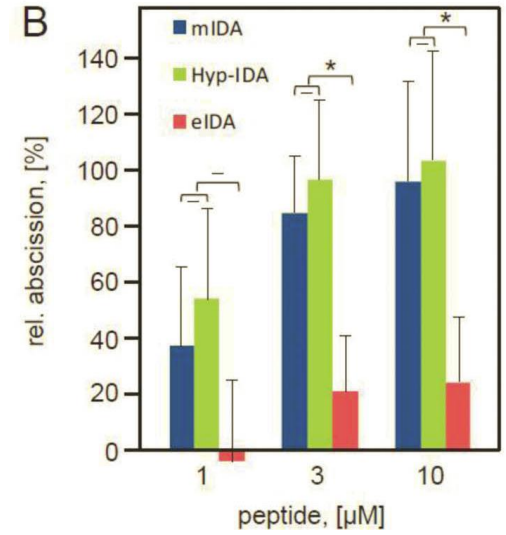



Fiume & Fletcher 2012

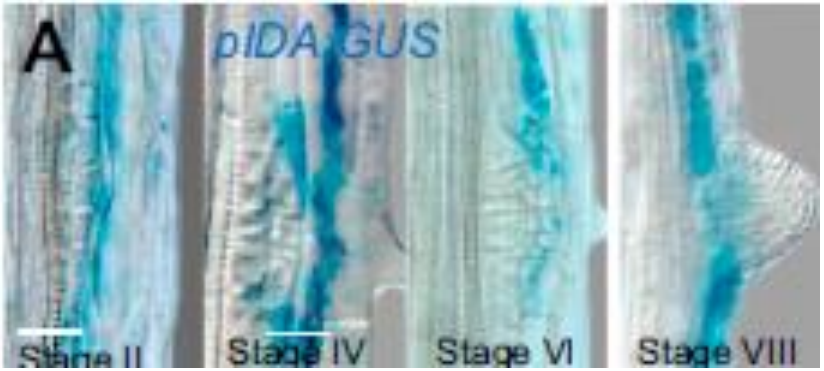


# IDA peptide

- INFLORESCENCE DEFICIENT IN ABCISSION (IDA)
- IDA is a 14-amino acid peptide that controls the separation step of floral organ abscission (Kumpf *et al.* 2013)
- A proline can be hydroxylated (Hyp) in the 9-position (Stührwohldt *et al.* 2018)
- Controls cell separation in lateral root emergence




Stührwohldt *et al.* 2018




Kumpf *et al.* 2013

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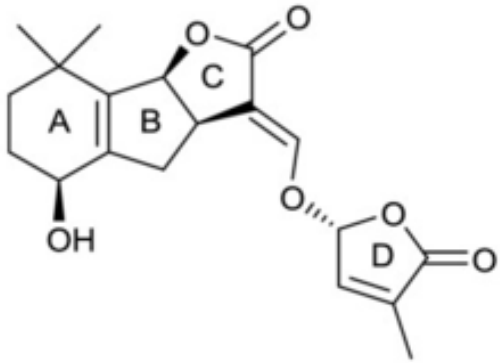
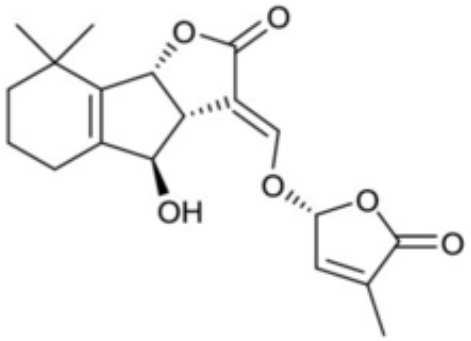
# Strigolactones

- Carotenoid-derived terpenoids
- First discovered from exudates of the parasitic plant seeds of Striga and Orobanche
- Stimulates arbuscular mycorrhizal fungi interaction with roots
- Mycorrhizal cell proliferation can be induced at concentrations of $10^{-13}$ M (Besserer *et al.* 2006)




Strigol




Orobanchol

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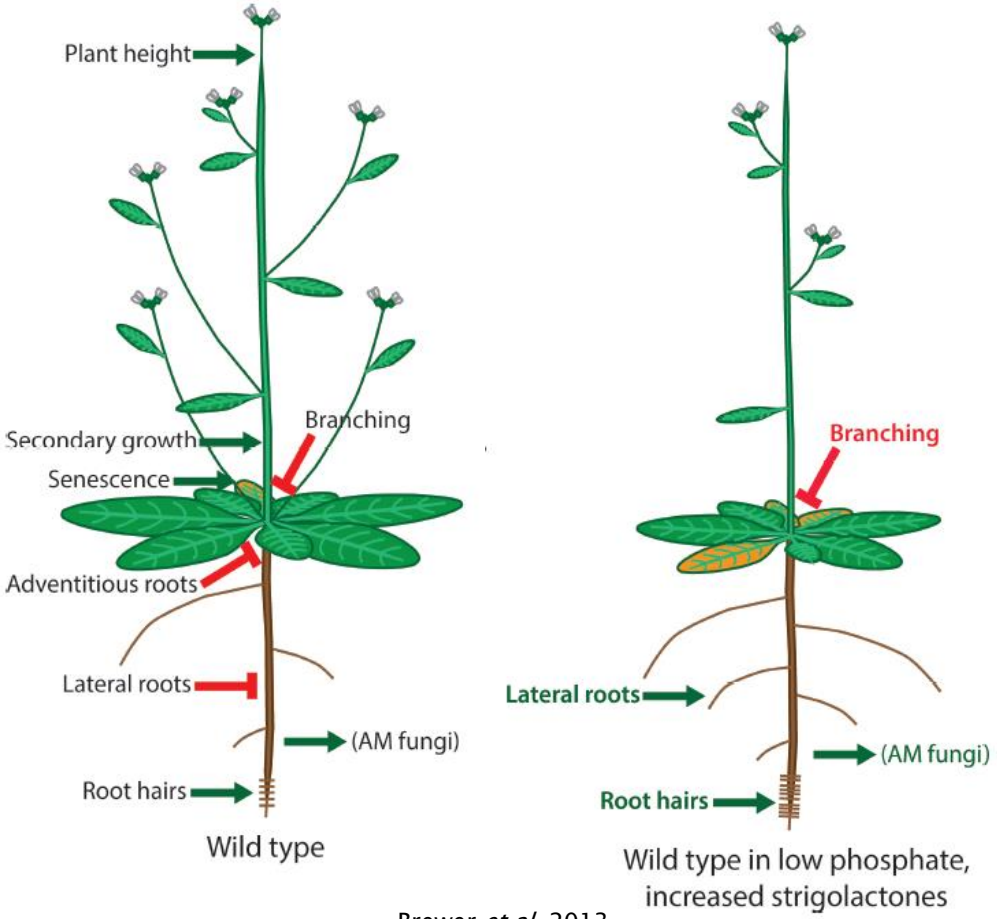
# Effect of Strigolactones

- Two studies showed they inhibit shoot branching in pea, rice, and arabidopsis (Gomez-Roldan *et al.* 2008; Umehara *et al.* 2008)
- Lateral apical secondary shoot's are inhibited
- This discovery makes Strigolactones major players in apical dominance




Brewer *et al.* 2013

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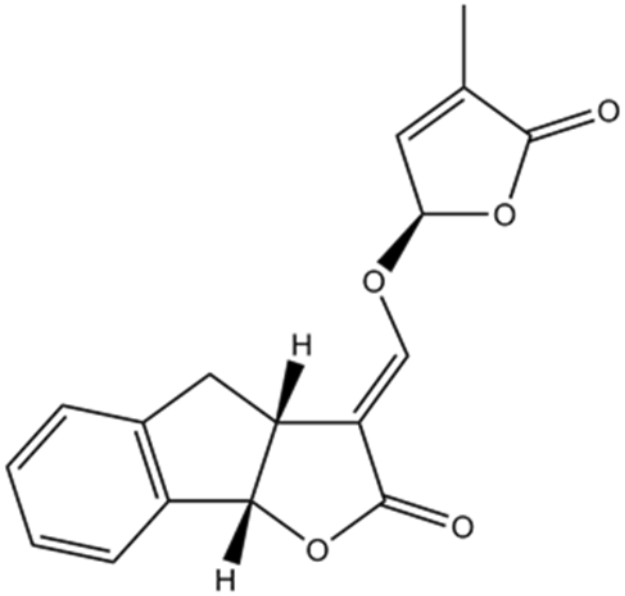
# GR24 (Prod. No. G3324)

- (+/−)-GR24 is the model analogue of strigolactone
- GR24 acts downstream of Auxin (Brewer *et al.* 2009)
  - GR24 repressed axillary bud growth without apical auxin
- GR24 repressed axillary bud growth when polar auxin transport was inhibited with NPA (Brewer *et al.* 2015)
- This suggests Strigolactones OR Auxins can maintain apical dominance




GR24

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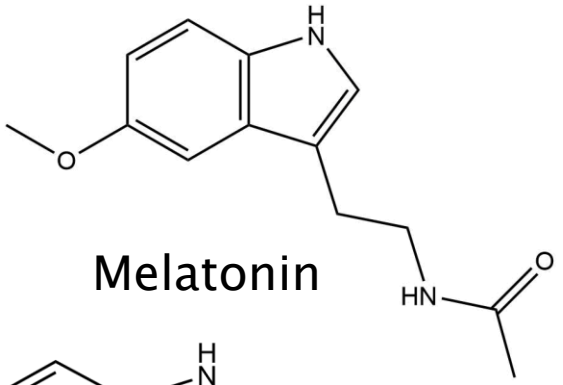
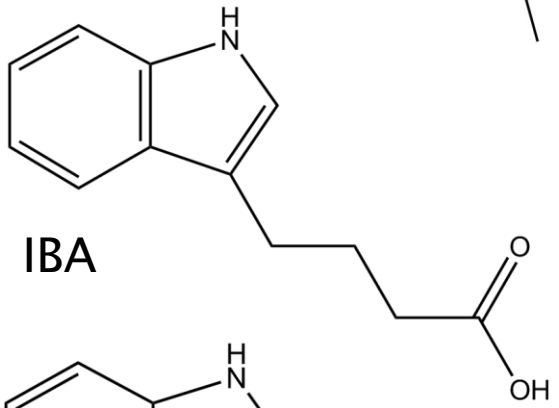
# Melatonin (Prod. No. M5520)

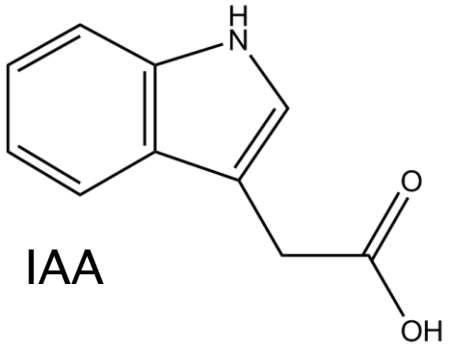
- Auxin-like, containing an indole ring
  - Promotes growth at μM but inhibits at higher concentrations (Arnao & Hernández-Ruiz 2014)
  - Similarly it is light sensitive in solution/media
- Lateral rooting is generally enhanced (Erland *et al.* 2015)
  - Primary rooting can be inhibited
- Antioxidant that can induce cold tolerance (Li *et al.* 2017)
- Balances with Serotonin similar to Auxin-Cytokinin




Melatonin




IBA




IAA

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# Stability of Cytokinins/Auxins

- Our work with Adenine-based cytokinins
  - Importance of solvent choice
  - Autoclavability
  - Crystallization & Freeze Thaw studies
- Recent work on Thidiazuron stability
- IBA/IAA stability

In Vitro Cell.Dev.Biol.—Plant (2016) 52:1–9
DOI 10.1007/s11627-015-9734-5

GROWTH REGULATORS

## Stability of adenine-based cytokinins in aqueous solution

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**Abstract** Since the isolation of the first cytokinin almost 60 yr ago, cytokinins have become critically important for ornamental and agricultural crops in plant tissue culture. Despite the extensive research on this class of compounds, little information is available on the chemical stability of cytokinins in solution or following an autoclave cycle with Murashige and Skoog (MS) basal medium. This work describes the stability in aqueous solutions of five widely used adenine-based cytokinins: trans-zeatin (tZ), 6-(γ,γ-dimethylallylamino) purine (2iP), kinetin, benzyladenine (BA), and *m*-topolin. High pressure liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS) were used to quantify and identify their degradation. BA, kinetin, 2iP, and *m*-topolin were stable at 1.0 mg $mL^{-1}$ in 0.05 N KOH, with no statistically significant concentration changes ($p > 0.05$) after 90 d of storage at temperatures of −20°C, 2–6°C, or 25°C. The cytokinin tZ was used as a model compound to evaluate stability under alkaline and acid conditions as well as after repeated freeze-thaw cycles. Trans-zeatin retained >90% of the initial concentration of 1.0 mg $mL^{-1}$ when dissolved in 0.01 N KOH and stored at −20°C and 2–6°C for 90 d, with only the 2–6°C temperature treatment showing a statistical significant concentration change ($p = 0.03$). The 1.0 mg $mL^{-1}$ tZ solution in 0.01 N KOH was stable through six repeated freeze-thaw cycles over 90 d without any significant change in concentration compared to the initial freeze-thaw. Yet, tZ showed highly significant concentration changes when dissolved at 50 mg $mL^{-1}$ and 0.5 N KOH. All of these adenine-based cytokinins showed exceptional stability following an autoclave cycle at 121°C, 110 kPa for 30 min when in solutions of 1.0 mg $mL^{-1}$ in 0.05 N KOH, with no significant degradation detected. Trans-zeatin was also found to be stable after one autoclave cycle with 1× MS-basal salts.

**Keywords** Cytokinin · Stability · HPLC · FTIR · Mass spectrometry

**Electronic supplementary material** The online version of this article (doi:10.1007/s11627-015-9734-5) contains supplementary material, which is available to authorized users.

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### Introduction

Cytokinins are a class of plant-growth regulators that were discovered because of their ability to enhance cell division in plant-tissue culture (Miller *et al.* 1955). Since the discovery of cytokinins, their disruption of apical dominance (Wickson and Thimann 1958), their biosynthesis from tRNA (Skoog and Armstrong 1970; Letham and Palni 1983) and *de novo* (Takei *et al.* 2001; Takei *et al.* 2004), as well as their role in plant development (Werner *et al.* 2003; Besnard *et al.* 2014) and signal transduction (Kakimoto 1996; Brandstatter and Kieber 1998; Hwang and Sheen 2001) have become well-defined processes. The use of cytokinins for the maintenance of the shoot apical meristem (Shani *et al.* 2006), and their *in vivo* metabolism (Mok and Mok 2001) in general have also become better understood. In spite of our knowledge of these processes at the cellular level, little is known about the chemical stability of cytokinins in solution or their physical stability during storage or an autoclave cycle. Though some phenylurea derivatives (e.g., thidiazuron, 4-CPPU) display

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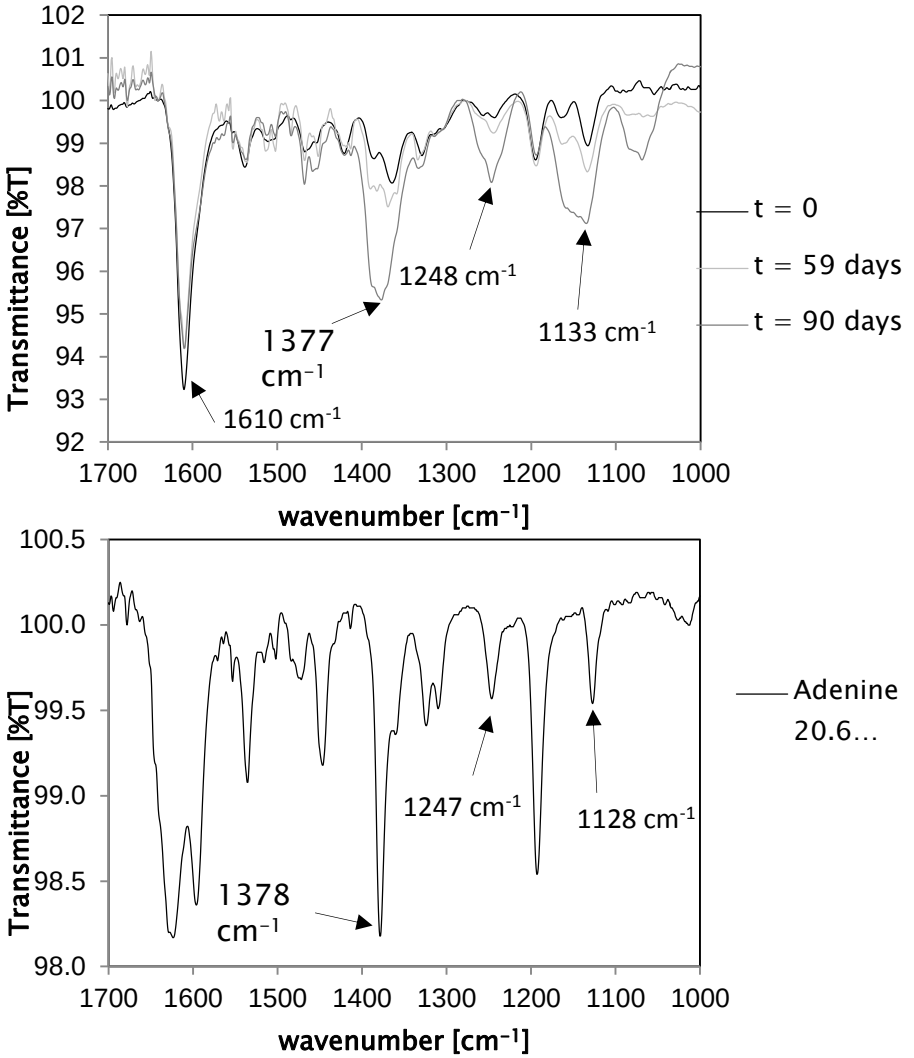
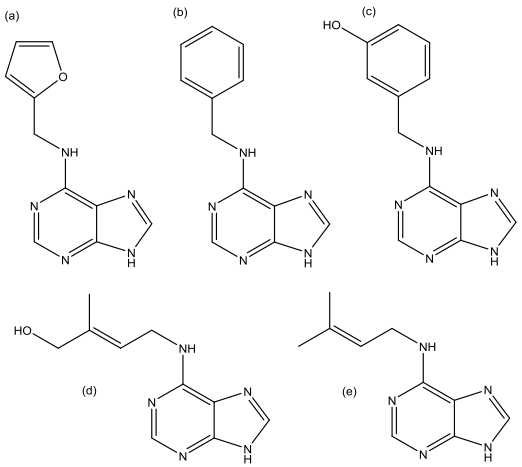
# Needed High concentrations for FTIR-ATR

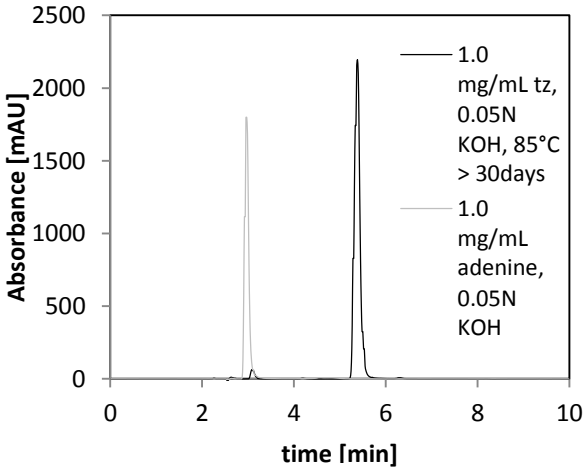
- Initially FTIR-ATR was used to watch for structural changes
  - Zeatin (50 mg/mL) in **0.5N KOH** at 40°C
- Evidence the isoprenoid side chain was broken and adenine formed
- 20% of Zeatin stored at -20°C degraded after 21 days
- But we wanted to look at lower stock solution concentrations

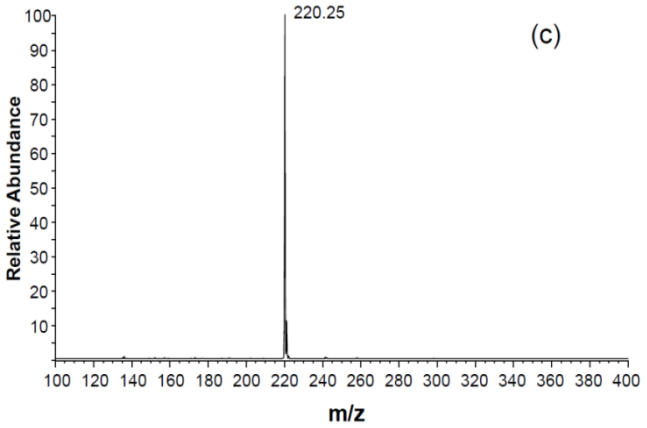


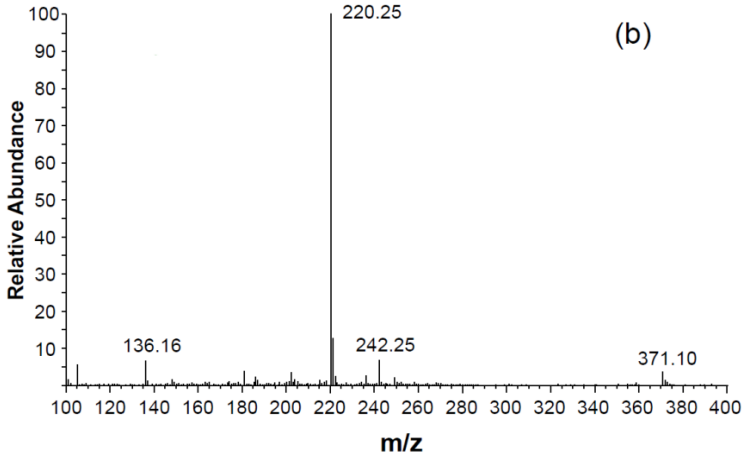
# HPLC-MS

- Developed an HPLC-MS method to distinguish between cytokinins and degradation products
- Adenine was a major degradation product
- Loss of absorbance at 270 nm accompanied degradation
  - With Zeatin the adenine ring is the main source of absorbance at 270 nm
  - Implied that adenine was damaged

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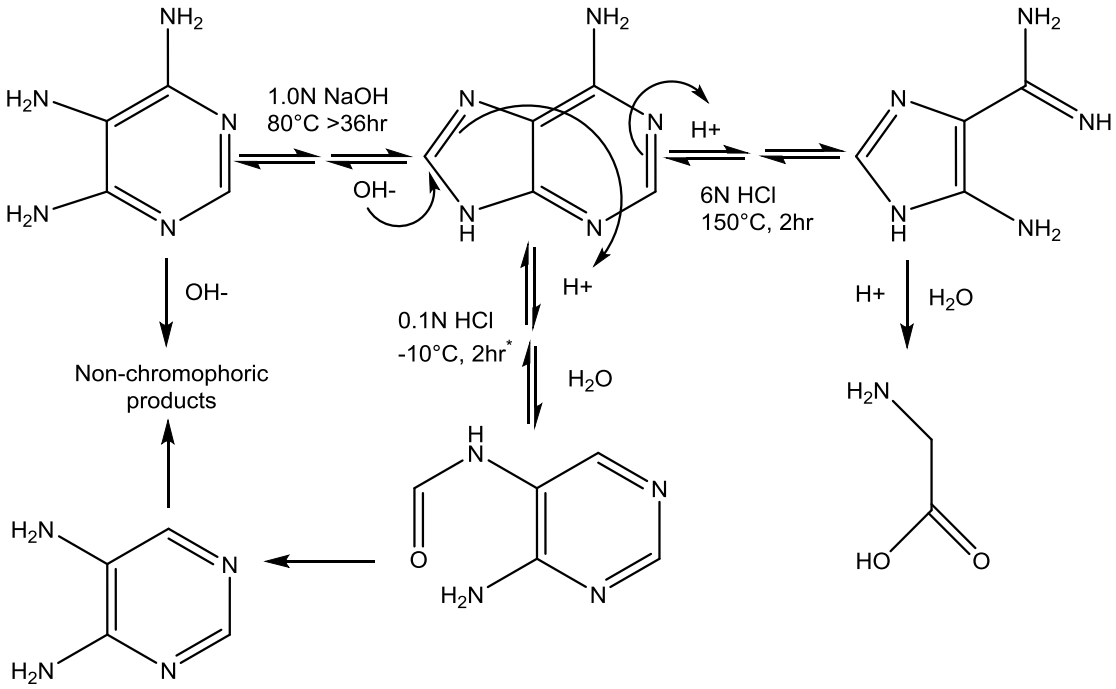
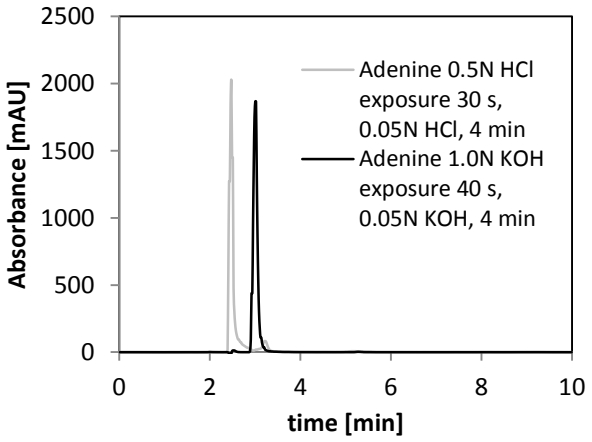
# Adenine Stability–Solvent choice

- Adenine was known to be stable at 100°C for 1hr in 1.0N KOH (Jones *et al.* 1966)
  - 0.5N KOH in the FTIR was likely breaking the chain & adenine
- Exposure to 0.5N HCl even for 30 seconds changed adenines HPLC retention time
- Acids never should be used
- But low base concentrations appear to be ok based on HPLC

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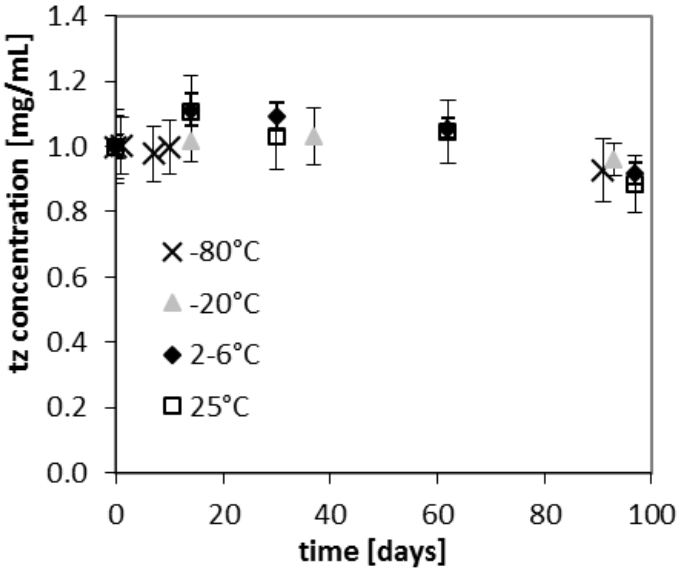
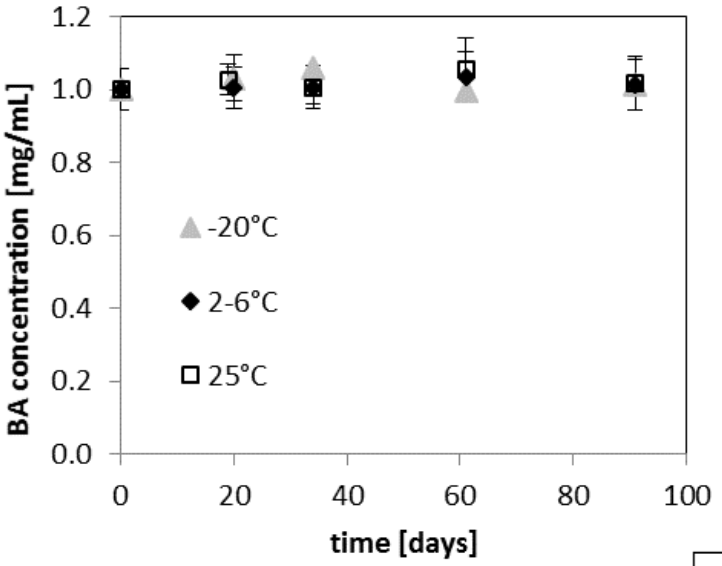
# Stability Profiles

- All of the adenine-based cytokinins stored temperatures studied did not see statistically different concentration changes
- Only Zeatin at 2-6˚C at 90 days was statistically different

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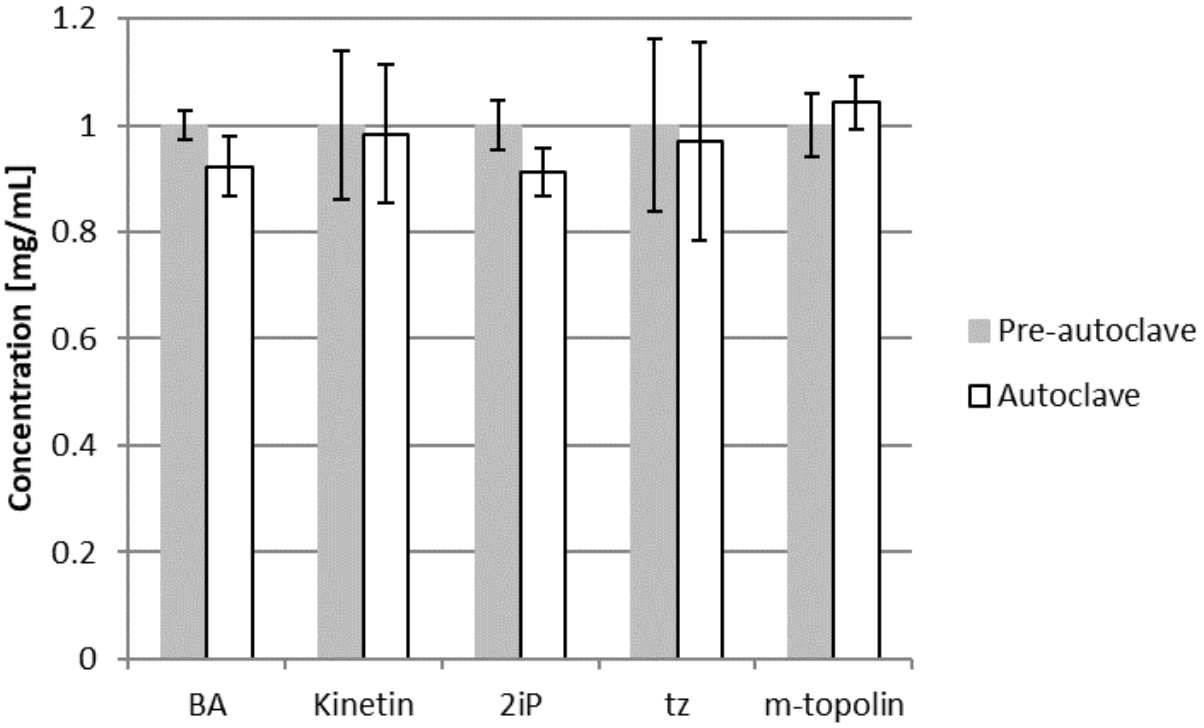
# Adenine-based Cytokinins are Autoclavable!

- Autoclaved 1.0 mg/mL solutions at 121°C at 1.1 bar for 30 min
- Autoclaving tZ in MS media did not show any concentration change

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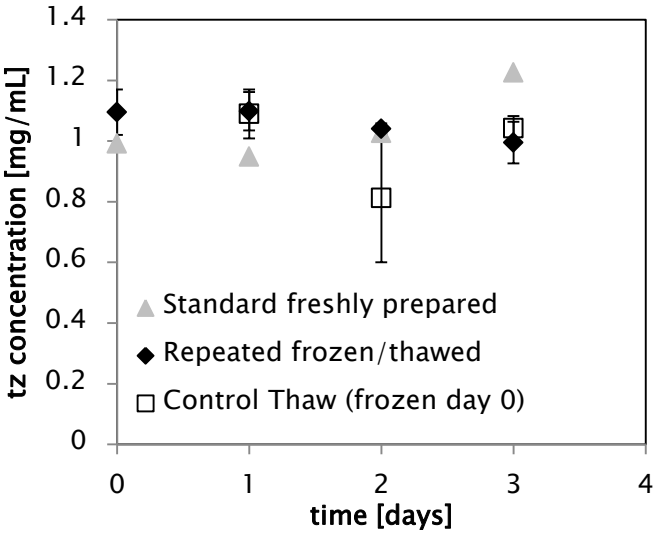
# Crystallization & Freeze Thaw

- Crystallization occurred with Phenyl-ring substituted adenine-based cytokinins (Kinetin, *m*-Top, BA) solutions
  - Rapid cooling (~30°C to 2-8°C)
  - Long term storage of higher concentrated solutions (>1.0 mg/mL)
  - Higher frequency at lower KOH concentrations (10 mM)
- Crystallization was sporadic, but to overcome
  - Increasing to 50 mM KOH maintained solubility




Zeatin was stable to multiple-freeze thaws (-20°C – 22°C)

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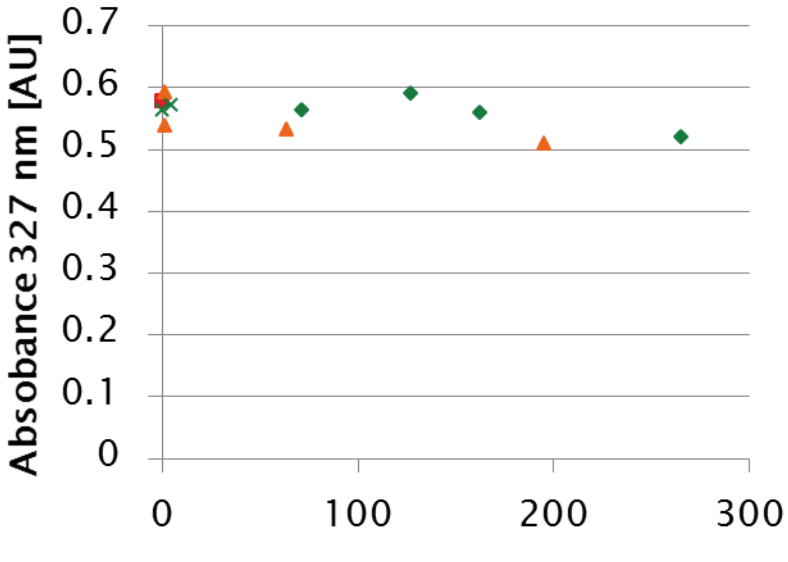
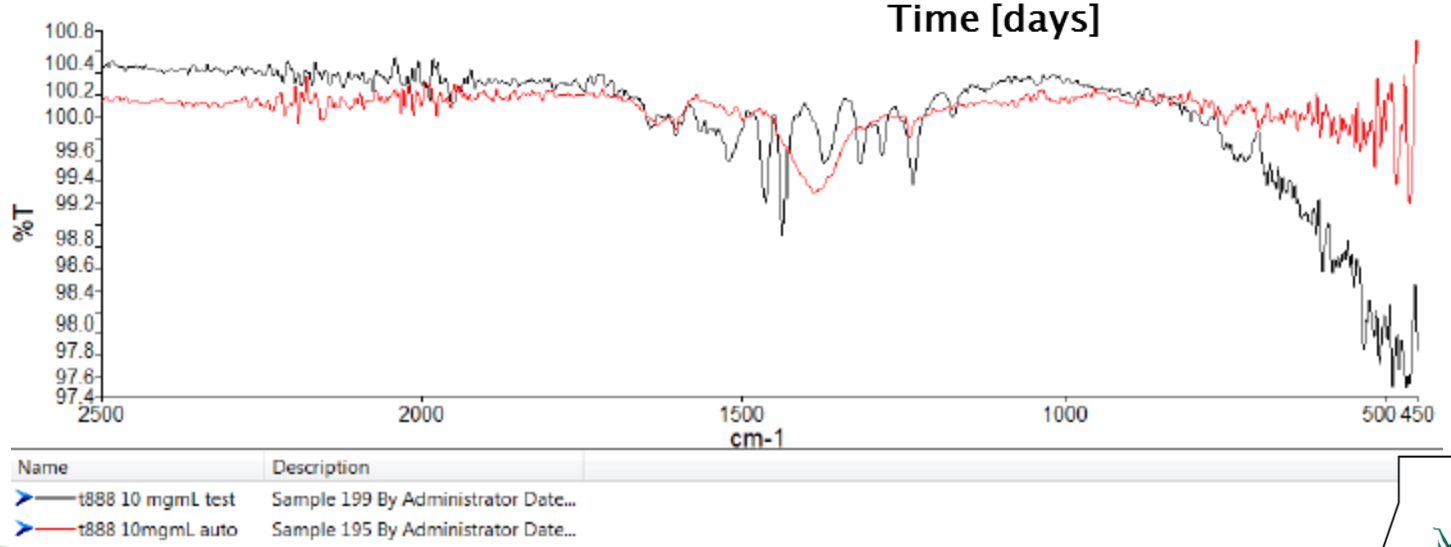
# Thidiazuron (TDZ) stability

- We knew TDZ was stable in 0.1N KOH for at least 9 months
- Autoclaving for 30 min at 121°C and 1.1 bar showed significant FTIR changes

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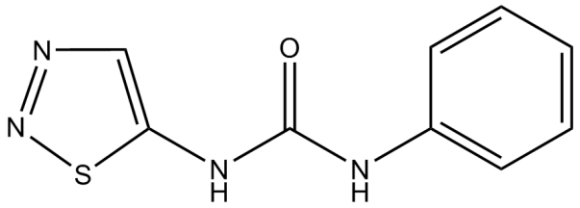
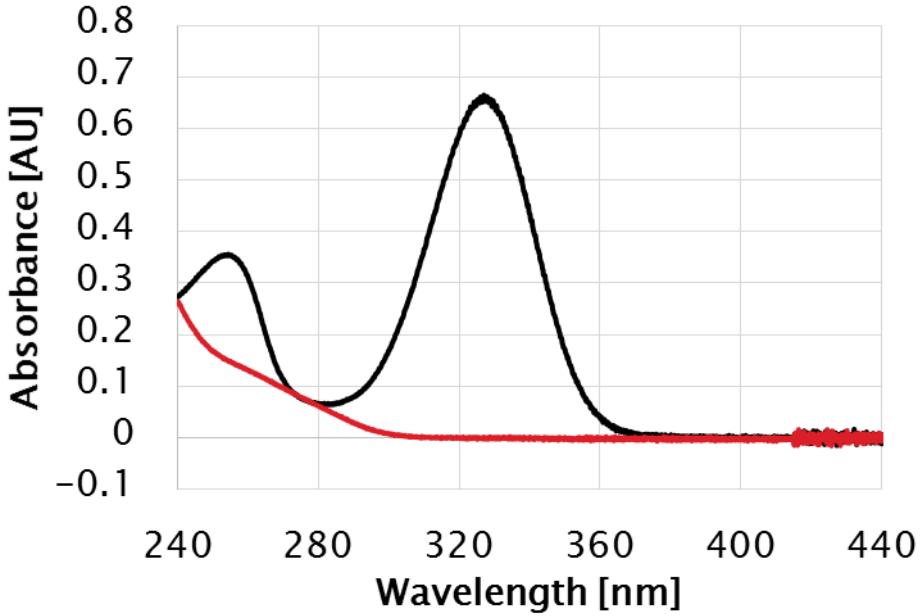
# TDZ Stability

- UV-Vis spectroscopy supported the FTIR finding that significant changes were occurring
- Autoclaving completely destroyed the absorbance at 327 nm
- We are working on Mass Spectrometry currently




TDZ

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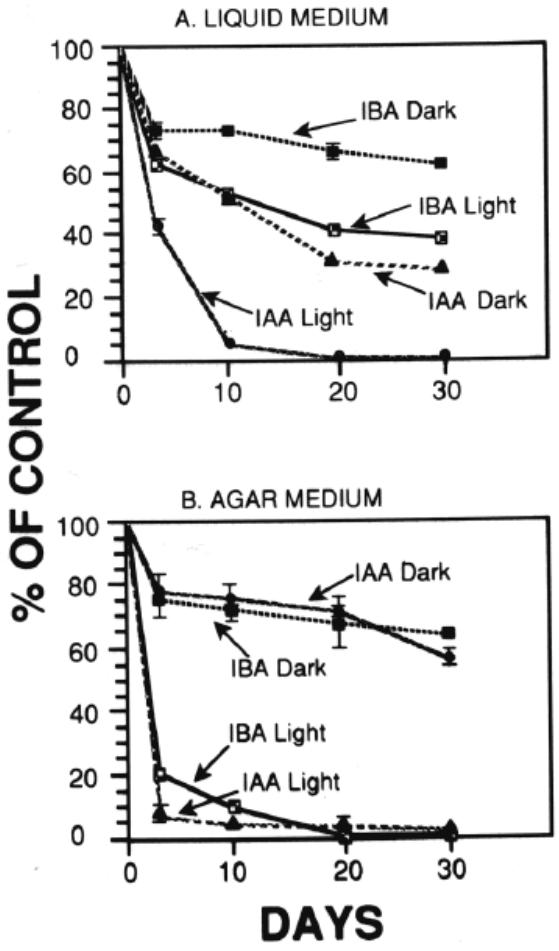
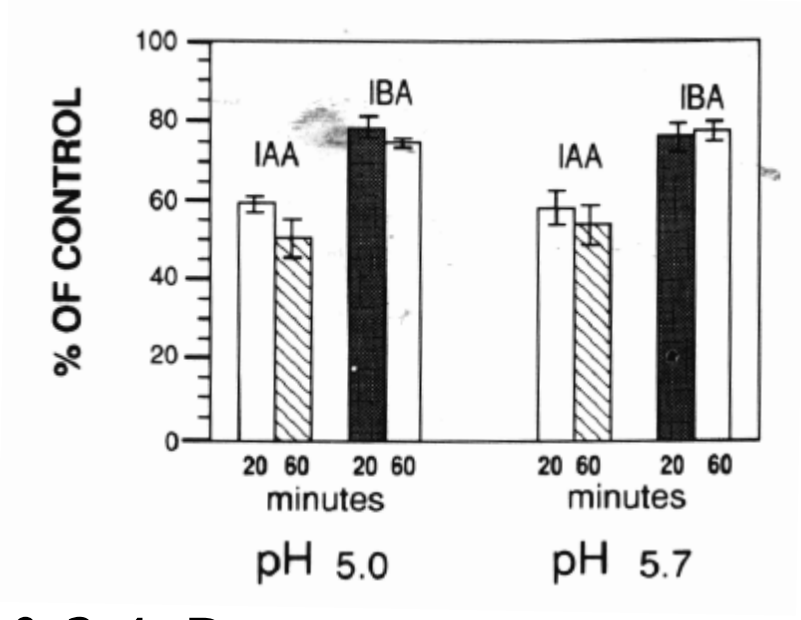
Plant Growth Regulators

# Auxin Stability




- NAA & 2,4-D are autoclavable and light stable
- Nissen & Sutter (1990) established the first studies on the stability of PGR's
- IAA & IBA are autoclavable but they lose some activity during a cycle (Above)
- IAA & IBA are also not as light stable when dissolved (Left)

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# Questions

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