QUARTERLY REPORT

JULY 1, 2017 – SEPTEMBER 30, 2017

Kentucky Tobacco Research & Development Center

College of Agriculture, Food and Environment

Kentucky Tobacco Research and Development Center

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TABLE OF CONTENTS

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EXECUTIVE SUMMARY

Introduction

The legislation (KRS 248.510 - 248.580) which provides funds in support of the research programs at the Kentucky Tobacco Research and Development Center (KTRDC) requires that a quarterly research report be submitted to the Kentucky Tobacco Research Board (KTRB) and the Legislative Research Commission.

The overall reporting plan is:

January 1	-	March 31:	Selected topics
April 1	-	June 30:	Selected topics
July 1	-	September 30:	Selected topics
October 1	-	December 31:	Annual comprehensive report

As required by KRS 248.570, a financial report covering expenditures for the relevant proportion of the July 1, 2017 – September 30, 2017 fiscal year is included in this report.

The news and research publications provided in this quarterly report are a representative selection of the Center's output. For a full description of all KTRDC research and activities please refer to the KTRDC Annual Report.

Quarterly News

At the September KTRB meeting, the discussion centered on recent FDA announcements for proposed product standards for NNN in smokeless tobacco products (January 2017) and nicotine levels in combustible cigarettes (July 2017). The ability to set standards has always been a significant part of the Family Smoking Prevention and Tobacco Control Act, but these are the first of potentially many proposed standards that are expected from the FDA. As part of the nicotine announcement, the FDA touted the concept of a "Risk Continuum" for tobacco products with combustible products considered as higher risk and other tobacco products, including vapor/e-cigarette products, smokeless tobacco products, and pharmaceutical nicotine products (patches, gums, etc.), defining a continuum of relative risk for chronic disease in consumers. This approach is not accepted by all of the tobacco research community (including many involved with human health research), so the proposed standards and emphasis on transitioning smokers to next-generation nicotine delivery products has generated considerable debate. For KTRC, this debate guides our research program, because part of our focus is on projects that support science-based policy decisions.

An example of how KTRDC research impacts both public health and tobacco agriculture was highlighted at the KTRB meeting. The University of Kentucky LC

seed screening program follows a protocol developed by KTRDC scientist Anne Jack, and the program continues to be coordinated and implemented through the KTRDC analytical lab to screen all foundation seed for plants that convert high levels of nicotine to nornicotine. The high-converter plants are eliminated from the seed production process which lowers nornicotine levels in both burley and dark tobacco produced in Kentucky and throughout the world. Nornicotine is the precursor for NNN during curing, which is the focus of the first proposed FDA standard. There continues to be a considerable amount of KTRDC research dedicated toward lowering NNN levels in tobacco products. In addition, several new research initiatives are meant to help Kentucky tobacco producers meet the proposed FDA standards, and include developing varieties that are better suited for next-generation tobacco products. These programs were outlined at the KTRB meeting and were also presented to the state House and Senate Agriculture committees during their September joint session. These state legislative committees also visited KTRDC and toured the building as part of the CAFE Ag Roundup activities in October.

The KTRDC Center for Tobacco Reference Products (CTRP) has also been very active this year in conducting three proficiency studies and producing several new tobacco reference products. The first proficiency test, CIG-2017A, included nine smoking parameters (formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, 2-butanone, n-butyraldehyde, and Puff Count) using the 1R6F reference cigarette as the proficiency test material smoked in both the ISO and the Health Canadian Intense smoking regimes. This test also included the determination of physical properties of the test material: resistance to draw, total ventilation, filter ventilation, pressure drop (closed), tobacco weight, cigarette weight, air permeability, firmness, circumference, length – cigarette, length – filter plug, and length – tipping paper. Eighteen linear Proficiency Test Kits and seven rotary kits were sold to laboratories throughout the world. Of these 25 total kits sold, data was submitted for 18 kits. This round is complete and the final report was released in September 2017. The second round of proficiency testing, CIG-2017B, tested basic smoking parameters such as TPM, nicotine, CO, water, NFDPM (Tar), and Puff Count using the 1R6F reference cigarette as proficiency test material smoked in both the ISO and the Health Canadian Intense smoking regimes. This test also included the determination of physical properties of the test material as for CIG-2017A. Eighteen linear Proficiency Test Kits and 10 rotary kits were sold. Data was received for 25 of the kits. The interim report has been released and the final report is being drafted. The third round, CIG-2017C, tests for NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNN (N-nitrosonornicotine), NAT (N-nitrosoanatabine), NAB (N-nitrosoanabasine), and BaP (Benzo[α]pyrene), TPM, Puff Count and physical parameters. Seventeen proficiency test kits have been sold and orders continue to be filled for this product. Participants will have until mid-December 2017 to submit their data with the interim and final reports being issued in 2018. The CTRP continues to provide reference tobacco products to researchers throughout the world; we recently added two cigar fillers to our reference tobacco program and are in the process of certifying constituent levels in three new smokeless reference products

(loose leaf, moist snuff, and snus). The CTRP program is unique to KTRDC and is a vital tool in the effort to establish science-based research on tobacco products.

As an example of the usefulness of CTRP programs and reference products, Report #1 highlights the science being developed and compared in various labs throughout the world (in this particular case for methods to measure nicotine in tobacco products). KTRDC actively participates in such research and also provides reference products for collaborative studies like the CORESTA report summarized here.

Summary of Selected Research Topics

Report #1 "2016 Collaborative Study on Nicotine in Tobacco Products". G. Gillman, H. Ji and M. Morton.

Several KTRDC researchers are involved in CORESTA (Centre de Coopération pour les Researches Scientifiques Relatives au Tabac), which is an international tobacco research organization that has 148 member institutions worldwide. Ms. Huihua Ji, the principal scientist from the KTRDC tobacco analytical laboratory, participates in many CORESTA studies that are meant to help establish the science of tobacco constituent measurement. Ms. Ji is a co-author for this collaborative CORESTA report that presents the results of a study to measure nicotine in tobacco products. This study is particularly relevant given the proposed new product standard for nicotine in cigarettes, and suggests that the method used by the participants is appropriate for the analysis of both tobacco and smokeless tobacco products. The CORESTA Routine Analytical Chemistry Sub-Group and the Smokeless Tobacco Subgroup routinely evaluate reference tobacco products (including some provided by KTRDC) as part of their efforts to improve the chemical analysis of tobacco and tobacco products.

Report #2 "Challenges towards Revitalizing Hemp: A Multifaceted Crop". C. Schluttenhofer and L. Yuan.

Hemp has been an important crop throughout human history for food, fiber, and medicine, and it is now being touted as an important new crop opportunity for Kentucky farmers. Despite significant progress made by the international research community, the basic biology of hemp plants remains poorly understood, at best. Clear objectives are needed to guide future research to promote and support hemp as a feasible and economical agricultural crop. In this paper, two KTRDC scientists summarized the many desirable traits that require improvement, including eliminating seed shattering, enhancing the quantity and quality of stem fiber, and increasing the accumulation of phytocannabinoids. In addition, methods to manipulate the sex of hemp plants will likely also be important for optimizing yields of seed, fiber, and cannabinoids. The authors present some of their data on seed shattering and review the status of hemp production and research. The paper is the issue cover story of the popular journal Trends in Plant Science.

Report #3 "Cytokinin signaling promotes differential stability of type-B ARRs". T. Shull, J. Kurepa and J. Smalle.

The phytohormone cytokinin controls key aspects of plant growth and development, including agriculturally important processes like seed yield and the shelf life of produce. This paper offers new insight into how plants respond to cytokinin. The authors have found that high cytokinin concentrations have a different effect on different members of a family of proteins that promote the cytokinin response. In particular, they find that high cytokinin concentrations increase the abundance of one family member while decreasing the abundance of another. This discovery provides a better understanding of the cytokinin signaling mechanism and provides new tools and avenues to alter the cytokinin regulation of crop species with the aim of improving agricultural yields.

Report #4 "The miRNAome of *Catharanthus roseus*: identification, expression analysis, and potential roles of microRNAs in regulation of terpenoid indole alkaloid biosynthesis". E. Shen, S. Singh, J. Ghosh, B. Patra, P. Paul, L. Yuan and S. Pattanaik.

MicroRNAs (miRNAs) comprise a major class of endogenous, non-coding small regulatory RNAs that are 21 to 24 nucleotides in length. They are present in a variety of organisms from algae to plants. miRNAs are involved in the regulation of numerous biological processes, including plant hormone homeostasis, root development, leaf morphogenesis, flower development, and embryogenesis. However, information on the involvement of miRNAs in regulating specialized metabolite (natural products) biosynthesis is limited. *Catharanthus roseus*, the Madagascar periwinkle, produces a number of pharmaceutically valuable, bioactive terpenoid indolealkaloids (TIAs). Using small RNA-sequencing, Dr. Yuan and his team identified 181 conserved and 173 novel miRNAs (cro-miRNAs) in *C. roseus* seedlings. Genome-wide expression analysis revealed that a set of cro-miRNAs are differentially regulated in response to the phytohormone methyl jasmonate. Moreover, they demonstrated that a miRNA-regulated ARF, CrARF16, binds to the promoters of key TIA pathway genes and represses their expression. The *C. roseus* miRNAome reported in this paper provides a comprehensive account of the cro-miRNA populations, as well as their abundance and expression profiles in response to jasmonate. In addition, the findings underscore the importance of miRNAs in posttranscriptional control of the biosynthesis of specialized metabolites. This type of research is valuable in KTRDC's efforts to identify and optimize plants for the production of commercially useful compounds from plants.

Routine Analytical Chemistry Sub-Group
Smokeless Tobacco Sub-Group

Technical Report

2016 Collaborative Study on Nicotine in Tobacco Products

February 2017

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Table of Contents

1. Summary

CORESTA Scientific Commission requested the CORESTA Routine Analytical Chemistry Sub-Group (RAC) to develop a new CORESTA Recommended Method (CRM), without the need for standard addition studies, for analysis of nicotine in all tobacco products. From 2013 to 2016, RAC and the Smokeless Tobacco Sub-Group (STS) conducted a series of collaborative studies for the determination of nicotine in tobacco and tobacco products including cigarette and cigar filler, and smokeless tobacco products. The RAC and STS eventually selected a gas chromatography mass spectrometry (GC-MS) method using methanol as the extract solvent and quinoline as the internal standard. Nineteen laboratories participated in the final collaborative study conducted in the Spring of 2016. The purpose of this study was to evaluate repeatability and reproducibility (r & R) values of the methodology and draft a new CRM for the determination of nicotine in tobacco and tobacco products. The study results confirm that the method is appropriate for the purpose and the results are suitable for inclusion into a CRM.

2. Introduction

In late 2015 to early 2016, the CORESTA RAC and STS conducted a collaborative study that included CORESTA Reference Products (CRPs) 1-3, three cigarette fillers, two ground cigar fillers and one mint flavoured moist smokeless tobacco (MST) product. The purpose of this study was to evaluate the repeatability and reproducibility for the stated GC-MS method.

The nicotine content of tobacco was determined by pre-treating a tobacco sample with 2N sodium hydroxide and then extracting it in methanol with quinoline as the internal standard. The resulting sample extract was analysed by GC-MS in the selected ion monitoring (SIM) mode with electron-impact (EI) ionization. Data analysis was in basic conformance with the recommendations of ISO 5725-2:1994 and ISO/TR 22971:2005. The raw data were obtained from 19 laboratories. Analyte levels were reported in units µg/g of tobacco on an as-is basis and converted to mg/g on an as-is basis for statistical analysis.

3. Organization

3.1 Participants

A list of the participating laboratories is provided in Table 1. The laboratories are listed in alphabetical order. The numerical laboratory codes used in this report do not correspond to the same order as shown in the table below. The lab codes used in this study start at number 2. This study was run concurrently with another study and laboratory 1 did not take part in this study. For consistency, the same laboratory codes were used for both the nicotine and minor alkaloids studies[1].

[1] CORESTA Technical Report: 2016 Collaborative Study on Minor Alkaloids in Tobacco Products – February 2017 [RAC-STS-055-CTR]

Table 1: List of Participating Laboratories

Participants
Altria Client Services LLC (ACE), United States
Altria Client Services LLC (LPSS), United States
C.I.T.Montepaz S.A., Uruguay
China National Tobacco Quality Supervision and Test Center, China
Enthalpy Analytical Durham, United States
Enthalpy Analytical Richmond, United States
Global Laboratory Services, United States
ITG Brands, United States
Japan Tobacco LTRC, Japan
KT&G Research Institute, Korea
Labstat International ULC, Canada
Liggett Group, United States
Philip Morris International, Brazil
Philip Morris International, Switzerland
PT HM Sampoerna Tbk, Indonesia
RJ Reynolds Tobacco Company, United States
Swedish Match, North America, United States
Swedish Match, Northern Europe, Sweden
University of Kentucky, United States

3.2 Protocol

3.2.1 Study Samples

Laboratories were responsible for procuring all reference and monitor samples prior to starting the study. Laboratories were requested to store the samples at approximately 4 °C upon receipt if the analyses would be conducted within one week or to store the samples at approximately -20 °C if the analyses would be delayed. The study was to be conducted from October 2015 through February 2016. Laboratories were requested to submit data by February 10, 2016. The final data, including re-checks, were received by April 2016. The samples are identified in Table 2. The two cigar filler samples were provided by Altria Client Services LLC while the mint moist smokeless tobacco sample was provided by the American Snuff Company.

Table 2: Sample Identification

Sample Description
CRP1 - Swedish style snus pouch (2009)
CRP2 - American-style loose moist snuff (2009)
CRP3 - American-style loose dry snuff powder (2009)
Cigar filler #1 - Flavoured Ground Cigar Filler
Cigar filler #2 - Dark Air-Cured Ground Cigar (Wrapper, Binder and Filler)
CORESTA Monitor 8 (CM8) test piece
1R6F - participants will remove the filler from the cigarettes
1R5F filler - filler was pre-ground and homogenized by the University of Kentucky
(Mint MST) - American-style loose moist snuff with Mint

3.2.2 Within Laboratory Sample Preparation

The laboratories were directed to remove the samples from cold storage at least 24 hours prior to testing and to not open the samples until equilibrated to ambient temperature. The following sample preparation procedures were to be followed:

- CRP1: Remove unit pouches from a single can. Cut the pouches in half and add the tobacco from the pouch to the extraction vessel and then add the pouch material.
- CRP2, CRP3: Samples should be analysed without further sample grinding. Aliquots may be removed from a single can after mixing the contents of a can.
- 1R6F: The filler from 20 cigarettes (1 pack) should be removed from the paper and filter materials, ground, and mixed before aliquoting.
- 1R5F filler and cigar fillers: The fillers were pre-ground and homogenized.

3.2.3 Sample Analysis and Data Reporting

Laboratories were requested to conduct three (3) replicate analyses for each sample. The replicates should be determined from independent tobacco extractions. Data were reported in units of µg/g, on an as-is basis and were converted to mg/g for analysis purposes.

3.2.4 Deviations

Laboratory Lab 6 and Lab 20 analysed only the tobacco for CRP1. The intact pouch was not analysed as requested in the protocol.

4. Data – Summary Descriptive Statistics

The full data set is listed in Table 3. The results are presented on an as-is basis, without correction for moisture. Each analysis includes three replicates. Raw data plots that include all replicates, without removal of outliers, are given in Appendix A. Outliers are discussed in 5.1. Data eventually dropped as outliers are included in Table 3, but were eliminated prior to r&R calculation.

Table 3: Full Data Set (results are presented on an as-is basis)

The nicotine values are provided on an as-is mg/g basis without correction for moisture.

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
2	1	17.49	23.26	33.61	15.29	14.68	25.45	9.73	8.90	13.47
	2	17.61	22.86	33.91	17.28	14.82	25.42	9.46	8.77	13.81
	3	17.32	22.62	33.43	15.80	14.59	25.55	9.54	8.80	13.81
3	1	15.88	18.99	24.76	10.09	14.36	21.96	8.91	8.31	12.48
	2	16.14	19.85	27.79	10.29	13.33	22.16	8.98	8.10	12.58
	3	15.00	19.81	27.99	10.48	13.29	21.49	8.71	8.15	13.13
4	1	15.83	18.42	27.30	10.72	13.07	21.92	8.63	7.56	12.57
	2	15.60	18.70	27.64	10.86	13.05	22.02	8.63	7.79	12.47
	3	15.60	18.85	27.82	10.97	12.97	22.24	8.50	7.73	12.56
5	1	16.27	20.40	30.04	10.75	13.91	20.57	8.77	8.14	12.67
	2	16.15	18.78	29.00	12.81	13.40	23.33	8.97	8.34	12.38
	3	16.16	18.95	28.70	9.94	13.82	23.55	8.96	8.48	12.96
6	1	16.80	18.09	27.21	9.58	NA	NA	8.14	7.84	12.12
	2	16.85	17.89	26.78	9.61	NA	NA	8.46	7.92	12.14
	3	16.91	17.82	27.19	9.76	NA	NA	8.38	7.88	12.19
7	1	15.97	19.33	27.67	10.26	13.23	22.93	9.12	8.11	13.15
	2	16.16	19.21	27.61	10.22	13.21	22.63	8.92	8.17	13.21
	3	16.01	19.16	27.88	10.52	13.63	22.58	8.80	8.26	13.10
8	1	15.50	18.10	26.97	10.37	13.40	23.11	8.67	7.82	12.69
	2	15.73	18.28	26.57	10.97	13.24	22.91	8.00	8.27	12.69
	3	15.92	18.49	26.72	9.87	13.45	22.54	8.38	7.99	12.58
9	1	15.96	NA	26.02	NA	NA	NA	NA	NA	NA
	2	16.27	NA	25.41	NA	NA	NA	NA	NA	NA
	3	16.21	NA	25.96	NA	NA	NA	NA	NA	NA
10	1	14.91	17.75	26.88	9.54	11.90	20.79	8.17	6.82	12.02
	2	14.67	17.48	27.45	9.87	12.02	21.46	7.90	7.18	11.74
	3	14.66	17.62	26.31	9.70	12.35	20.72	7.81	6.96	11.95
11	1	15.81	18.60	28.23	10.08	12.72	22.64	8.91	7.92	12.08
	2	15.76	19.04	27.97	9.87	12.63	22.55	8.99	8.15	12.17
	3	15.75	18.71	28.17	9.85	12.60	22.56	8.70	8.02	12.22
12	1	14.39	16.50	26.14	10.42	12.43	21.63	7.92	7.18	11.65
	2	14.61	16.45	25.69	10.55	12.17	21.78	7.85	7.42	11.56
	3	14.35	16.75	25.71	9.81	13.10	21.49	8.03	7.10	11.33

Lab	Rep	1R5F	1R6F	CM8	CRP1	CRP2	CRP3	Cigar Filler #1	Cigar Filler #2	Mint MST
13	1	15.95	19.37	27.94	10.18	13.04	22.47	9.01	8.20	13.49
	2	15.96	19.39	28.54	10.37	12.83	22.82	9.00	8.30	13.08
	3	16.14	19.70	28.14	10.43	13.25	22.92	8.88	8.44	13.07
14	1	14.67	17.99	28.43	11.62	13.15	22.11	8.63	7.47	11.96
	2	14.69	17.95	27.51	11.11	13.76	21.78	8.52	7.48	12.32
	3	15.21	17.99	30.08	11.62	13.42	21.89	8.26	7.74	12.32
15	1	NA	20.06	26.77	NA	NA	NA	8.38	7.16	NA
	2	NA	19.78	26.57	NA	NA	NA	8.32	6.79	NA
	3	NA	19.98	27.03	NA	NA	NA	8.47	6.91	NA
16	1	16.88	17.56	27.55	9.44	12.74	22.09	8.35	7.71	12.25
	2	17.05	17.75	27.85	9.29	12.66	22.24	8.19	7.95	12.33
	3	17.03	17.59	27.44	9.39	12.69	22.11	8.19	7.80	12.40
17	1	17.15	NA	28.14	11.49	13.36	23.25	NA	NA	NA
	2	17.66	NA	27.69	12.17	14.22	22.95	NA	NA	NA
	3	17.71	NA	27.98	11.00	14.86	23.05	NA	NA	NA
18	1	15.76	19.00	32.10	13.17	13.07	22.53	8.72	7.51	12.54
	2	16.05	18.39	29.47	13.51	13.35	24.46	8.57	7.59	12.41
	3	15.59	19.02	31.15	11.87	13.06	21.89	8.76	8.55	12.89
19	1	16.22	NA	26.77	8.24	11.66	20.65	NA	NA	NA
	2	17.34	NA	26.91	8.60	11.62	20.82	NA	NA	NA
	3	19.86	NA	27.49	8.43	11.05	20.61	NA	NA	NA
20	1	16.74	13.82	26.62	9.22	12.51	21.17	7.46	6.99	11.23
	2	17.24	12.88	26.14	9.29	12.35	20.64	7.37	7.04	11.42
	3	17.21	12.90	26.24	9.23	12.28	20.41	7.42	6.76	11.30

NA indicate s that the laboratory did not provide data for that sample.

5. Data – Statistical Analysis

A statistical analysis was conducted in basic conformance with ISO 5725-2:1994 and ISO/TR 22971:2005. A summary of the results from outlier detection and the calculated results for repeatability (r) and reproducibility (R) are given below in sections 5.1 and 5.2, respectively. Raw data plots that include all replicates shown in Table 3, prior to removal of outliers, are given in Appendix A.

5.1 Exclusion of Outliers

Procedures outlined in ISO 5725-2:1994 and ISO/TR 22971:2005 were generally used for the exclusion of outliers. An adaptation of Levene's Test was used for eliminating laboratories with overly large repeatability standard deviations and Grubbs' Test was used to eliminate laboratories with outlying mean values.

ISO 5725(2) also recommends the use of Mandel's h and k plots. Mandel's h statistic is the same as the statistic used in Grubbs' Test. Similarly Mandel's k statistic, associated with within lab standard deviation, is statistically equivalent to the c-value calculated in Cochran's Test ($k = \sqrt{n_{labs}}c$). However, the critical values associated with Mandel's h and k statistics do not make allowance for multiple testing and can therefore, give a false impression of statistical significance. Thus, Mandel's h and k statistics do not add fundamentally new information and as typically employed may lead to incorrect conclusions. For those reasons, we do not include Mandel's h and k plots.

The intent of ISO 5725-2:1994 is to eliminate outliers that exceed a 1 % critical value. This was accomplished by an adaptation of Levene's Test. Levene's Test is preferable to Cochran's Test, which is recommended in ISO 5725-2:1994, because of Cochran's Test's extreme sensitivity to deviations from normality. Grubbs' Test and an adaptation of Levene's Test were applied at the standard nominal 1 % significance level to determine outliers and the results are shown in Table 4. Levene's Test is mentioned in ISO/TR 22971:2005 as an alternative to Cochran's Test. However, Levene's Test does not directly apply without adaptation. For more details, see the footnote below[2].

The initial examination for outliers indicated that Lab 2 tended to give outlying results. For that reason Lab 2 was dropped from the analysis and the data were again examined for outliers.

Table 4: Outliers

Sample	Levene's Outliers Lab	Grubbs' Outliers Lab
1R5F	19	–
1R6F	–	20

The (–) symbol indicates an outlier was not detected.

5.2 Calculation of Repeatability and Reproducibility

After removal of outlying data based on numerical data consistency methods discussed above (Grubbs' Test and Levene's Test), the final repeatability and reproducibility (r & R) results were calculated and are shown in Table 5. It should be understood that the r & R results reflect both laboratory variability and product consistency.

[2] Levene's Test is commonly used to determine if each of several subpopulations have the same variance. Since it was designed to test for overall differences, not to determine if the largest variance is significantly greater than the others, some adaptation is necessary to use the approach to eliminate laboratories whose within lab variation is too large. Levene's Test was adapted to this purpose by Morton, who presented the approach utilized in this report at the 2014 CORESTA Congress (Quebec, Canada, presentation ST28, October 14, 2014). Specifically, the approach taken here is a two-step process with a lab being eliminated as an outlier if both steps are statistically significant. First, Levene's Test was run at a nominal α-level of 0.02. Second a comparison of the largest variance to the remaining variances is carried out at a one-sided nominal level of α=0.01/number of labs. Dividing by the number of labs is to account for multiple testing, since it is not known *a priori* which lab will have the largest variance. Simulation studies were carried out by Morton and presented at the 2014 CORESTA Congress and these results demonstrated that this process has an overall α-level near 0.01 and is robust to deviations from normality.

Table 5: Repeatability (r) and Reproducibility (R) Limits for as-is Nicotine (mg/g)

Product	No. of Labs *	Mean	Repeatability		Reproducibility	
			r	r (%)	R	R (%)
1R5F - Ground Cigarette Filler	16	15,97	0,65	4,0%	2,44	15,3%
1R6F - Ground Cigarette Filler	14	18,56	0,90	4,8%	2,74	14,7%
CM8 - Ground Cigarette Filler	18	27,52	1,91	7,0%	3,83	13,9%
CRP1 - Pouched Snus	16	10,36	1,40	13,5%	3,18	30,7%
CRP2 - Loose Moist Snuff	15	12,98	0,93	7,2%	2,09	16,1%
CRP3 - Loose Dry Snuff Powder	15	22,10	1,66	7,5%	2,60	11,8%
Cigar Filler #1 - Flavored Ground Cigar Filler	15	8,46	0,42	5,0%	1,29	15,3%
Cigar Filler #2 - Dark Air-Cured Ground Cigar (Wrapper, Binder, and Filler)	15	7,73	0,58	7,4%	1,46	18,9%
Mint MST	14	12,37	0,50	4,1%	1,59	12,8%

* The number of laboratory data sets after removal of outliers.

6. Data Interpretation

The GC/MS method described herein was compared to the CRM 62 [3] methyl tert-butyl ether (MTBE) and hexane methods. The R (%) values are statistically significantly higher for the GC/MS method than for the CRM 62 MTBE method (p=0.002) but similar to the hexane method (p=0.14). A graph of the three methods' relative reproducibilities is given in Figure 1.

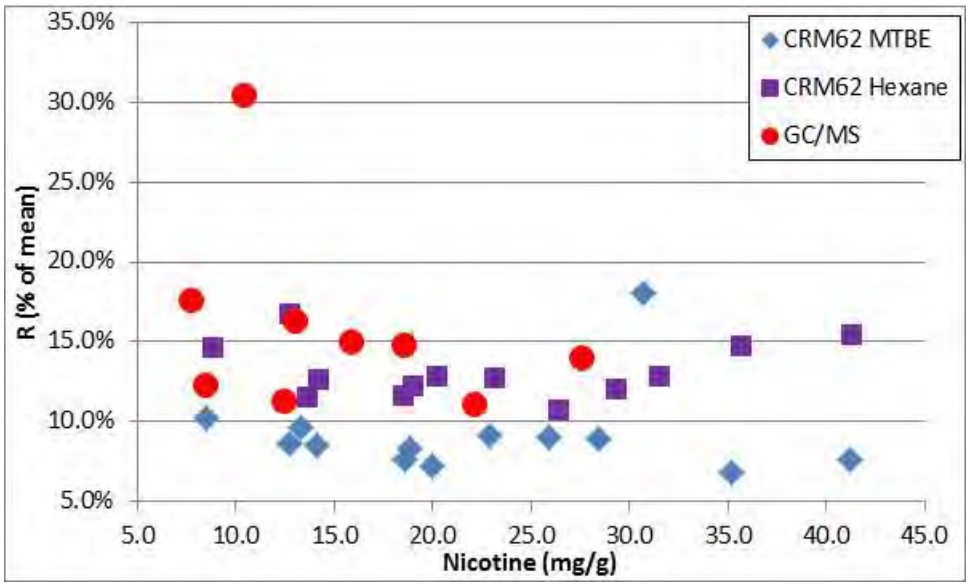


Figure 1: %R values, versus nicotine content, for this study compared to CRM 62.

[3] CORESTA Recommended Method No. 62 – Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis – February 2005 (Routine Analytical Chemistry Sub-Group).

The values for repeatability (r) and reproducibility (R) are shown in Table 5. The values for r & R demonstrate similar repeatability and reproducibility in both tobacco and smokeless tobacco. This table also includes values for % R for each sample, which range from approximately 12 % to over 30 %. These results indicate a similar level of variability for the determination of nicotine in both tobacco and smokeless tobacco, with the possible exception of CRP1, the only pouched product in this study. It was reported that the CRP-1 sample exceeded the recommended sample mass given in the protocol. In order to fall within the linear calibration range of the method, this sample required either dilution of the original sample or preparation of a new sample using less tobacco mass, which may explain the higher level of variability seen with this sample. The overall level of variability seen in this study was slightly higher than the CRM 62[4] collaborative study on nicotine in tobacco (Figure 1).

7. Recommendations

In 2015, RAC and STS conducted an inter-laboratory study for the determination of nicotine in tobacco and smokeless tobacco products. The overall level of variability seen in this study was slightly higher when compared to values reported in CRM 62 but this may be related to sample homogeneity or lack of experience with the method compared to CRM 62 and not the analytical method used for the current study. However, the study results for the pouched product, CPR1, showed higher variability as compared to the loose tobacco products in this study. In order to better accommodate pouched products, it is recommended that the method be modified to either extend the calibration range or to provide an alternative sample preparation procedure for pouched products. The results for this study showed similar r & R values for both tobacco and smokeless tobacco products which indicates that this method is appropriate for the analysis of both tobacco and smokeless tobacco products. No evidence was found for smokeless tobacco related method interferences such as interferences with nicotine or the internal standard and with flavour compounds present in these samples. The lack of interferences from flavour compounds eliminated the need for the standard addition experiments required in some other methods for the analysis of nicotine in smokeless tobacco. The results of this study were discussed during the CORESTA RAC and STS meeting held in April 2016, in Lausanne, Switzerland. The RAC and STS agreed that this method is fit for use for the determination of nicotine in tobacco and smokeless tobacco products.

[4] CORESTA Recommended Method No. 62 – Determination of Nicotine in Tobacco and Tobacco Products by Gas Chromatographic Analysis – February 2005 (Routine Analytical Chemistry Sub-Group).

APPENDIX A: Study Protocol

CORESTA ROUTINE ANALYTICAL CHEMISTRY SUB-GROUP
CORESTA SMOKELESS TOBACCO SUB-GROUP

Project Title: The Determination of Nicotine in Tobacco Products by GC-MS

Type of Document: Collaborative Study Protocol

Date: November 19th, 2015

Written by: Gene Gillman, Study Coordinator

Confidentiality Notice: All data submitted by participating laboratories will be coded and kept confidential.

1. Introduction

The overall objective of this project is to develop a CORESTA Recommended Method (CRM) for the determination of nicotine in tobacco and tobacco products. Tobacco products include cigarette and cigar filler, and smokeless tobacco products

2. Objective

The objective of this study is to calculate repeatability (r) and reproducibility (R) for the GC-MS method that is provided.

Note: Use of any method other than that specified will not support the study objectives and the data cannot be included.

3. Time schedule

Date	Activity
October 20, 2015	Laboratories state their intention to participate **and order study materials**
November 6, 2015	Finalize protocol and distribute
February 10, 2016	Laboratories submit results by this date
Spring 2016	Discuss results at RAC and STS meetings

Note: Although each participant should read the applicable methods to determine what supplies are needed in order to participate in the study, the following supplies may need to be ordered:

1. ISO Guide 34 certified reference standard Nicotine is recommended.
2. GC column: CAM column (30m x 0.25mm id x 0.25µm df) or equivalent polar, base-deactivated PEG column (e.g. Stabilwax-DB or Carbowax Amine).

4. Participating Laboratories

Following receipt of this protocol, the participating laboratories will confirm or notify the study coordinator of their intent to participate. Please include your complete company name and location.

5. Samples

The samples listed in Table 2 will be analyzed. Samples should be ordered from:

- CORESTA Reference Products (CRPs) - North Carolina State University
- 1R6F cigarettes, 3R4F cigarettes, and 1R5F filler - University of Kentucky 1R6F cigarettes, 3R4F cigarettes, and 1R5F filler - University of Kentucky can be ordered from **https://refcig.uky.edu/client/index.html**
- Mint Flavored US Moist Snuff – American Snuff Company
  Contact Dr. John Bunch to request samples.
  [redacted]
- Two processed Cigar Filler Samples – Altria
  Contact Dr. Karl Wagner to request samples.
  [redacted]

Cerulean Rockingham Drive Linford Wood East Milton Keynes MK14 6LY United Kingdom Tel: +44 1908 23 38 33 Fax: +44 1908 23 53 33 e-mail: sales@cerulean.com	Borgwaldt KC GmbH Spare Parts Department, Schnackenburgallee 15, D-22525 Hamburg Germany, Tel: +49 40 85 31 380 Fax: +49 40 850 56 00 e-mail: BKC@Borgwaldt.com

Participants may use an internal supply of these products assuming the samples have been stored unopened and under suitable conditions. It is critical that the CRPs have been stored at the recommended temperature of −20°C, or they should not be used.

Processed Cigar Filler samples

The samples should be stored at -20ºC for long term storage. At a minimum, the following equilibration procedure must be followed to ensure water re-equilibrates throughout the samples

1. Remove the samples from -20ºC.
2. Allow the unopened samples to equilibrate in the refrigerator for a minimum of 24 hours.
3. Allow the unopened samples to equilibrate at ambient conditions for a minimum of 1hour prior to opening.

Shake the ground filler samples vigorously prior to opening to break clumps and re-homogenize the samples.

All remaining samples should be retained in sealed containers at −20°C as they may be used for future collaborative studies for other analytes.

Table 2: Samples

CRP1 - Swedish style snus pouch	3 cans
CRP2 - American-style loose moist snuff	3 cans
CRP3 - American-style loose dry snuff powder	3 cans
Cigar filler #1- Pre-ground and homogenized by ALCS	1 container (~20g)
Cigar filler #2- Pre-ground and homogenized by ALCS	1 container (~20g)
CORESTA Monitor 8 (CM8) test piece	1 carton
1R6F filler - participants will remove the filler from the cigarettes	1 carton
1R5F filler - filler will be pre-ground and homogenized by the University of Kentucky	1 container
American-style loose moist snuff with Mint (Mint US MST)	4 cans

6. Analysis

6.1. Analytes: Nicotine will be determined in each sample. Use of ISO Guide 34 certified reference standards is recommended for this study. Laboratories may use their own source or purchase the suggested material listed above.

6.2. Methods: Participating laboratories should use the supplied GC-MS method for the determination of the analytes. Please keep in mind that data generated from methods

other than specified in this protocol do not support the study objectives and cannot be included in the study results.

Replicates and Sample Handling: Conduct three (3) independent replicate analyses for each sample. The replicates should be determined from independent tobacco extractions**.**

6.3. Sample preparation: Additional sample preparation requirements are listed in Table 3

Table 3: Sample Preparation Requirements

Product	Sample Preparation
CRP1	Remove unit pouches from a single can. Cut the pouches in half and add the tobacco from the pouch to the extraction vessel and then add the pouch material.
CRP2, CRP3, Mint US MST	Samples should be analysed without further sample grinding. Aliquots may be removed from a single can after mixing the contents of a can.
1R6F, Cigar Filler, CM8	The filler from 20 cigarettes (1 pack) should be removed from the paper and filter materials, ground, and mixed before aliquoting.
1R5F filler and Cigar fillers	The tobacco from these products has been pre-ground and homogenized. Therefore, these filler samples should be mixed in the container and used as-is.

7. Data Reporting:

Participating laboratories should use the embedded Excel document for data reporting. The analytes should be reported on an as-is and a dry weight basis. Other requested methodological details should also be reported in the data reporting sheet. The completed data sheet should be sent to the following:

Gene Gillman: [redacted]

Nicotine Analytical Method	Removed
Final Data Reporting Worksheet	Removed

8. Statistical Analysis

A statistical analysis in general conformance with ISO 5725-2:1994 and ISO/TR 22971:2005 will be conducted.

9. Presentation of the Results

The final output will be a presentation for discussion at the Spring 2016 RAC and STS meetings.

APPENDIX B: Analytical Method

DETERMINATION OF NICOTINE IN TOBACCO AND TOBACCO PRODUCTS BY GC-MS – METHOD FOR COLLABORATIVE TEST

1. INTRODUCTION

This document has been prepared for use by CORESTA RAC and STS members participating in the 2015 collaborative test for nicotine. The purpose of this method is to quantitatively measure the amount of nicotine in tobacco products by GC-MS using electron-impact (EI) ionization.

2. SCOPE

This method is applicable to the determination of the nicotine in a wide range of smokeless tobacco products, cigarette tobacco, and cigar tobacco.

3. PRINCIPLE

Nicotine is extracted from tobacco with aqueous sodium hydroxide (2N) and methanol using quinoline as an internal standard. After shaking for 30 minutes, the sample is filtered then analyzed by GC-MS using electron-impact (EI) ionization.

4. CHEMICALS

Standards		*CAS No.*
4.1	Quinoline	[91-22-5]
4.2	(-)-Nicotine (NIC)	[54-11-5]
Ancillary Chemicals		
4.3	Methanol (HPLC/ACS grade)	[67-56-1]
4.4	Sodium Hydroxide (NaOH) – 2N solution	[1310-73-2]
4.5	Water (Type 1 or HPLC grade)	[7732-18-5]

5. EQUIPMENT

In addition to the general glassware and apparatus found within a typical analytical laboratory, the following is required when performing this analytical method:

5.1 Capillary GC with MS detector and split inlet.

5.2 Analytical balance (with 0.1 mg accuracy)

5.3 Orbital Shaker (or equivalent)

5.4 Dispensette capable of delivering 40 mL (or equivalent)

5.5 Eppendorf repeater with disposable tips, or equivalent

Per sample replicate:

5.6 50-mL polypropylene centrifuge tube with screw-cap (or equivalent)

5.7 Syringe and syringe filter (0.45 µm, nylon)

5.8 Amber autosampler vial (2 mL) with PTFE screw-cap

6. STANDARDS PREPARATION

The following is an ***example*** of how to prepare stock standards and calibration standards. Different amounts and volumes can be used, if necessary, to prepare the standards, provided the concentration of the calibration standards prepared covers the anticipated concentration range of the samples.

6.1 Internal Standard

6.1.1 Quinoline Stock Solution (~ 50 mg/mL)

Accurately weigh approximately 1.25 g of quinoline into a 25-mL volumetric flask. Add a small amount of MeOH to dissolve then make to volume with MeOH. Mix well.

6.1.2 Internal Standard Spiking Solution (ISSS)

Accurately add 2.0 mL of the stock solution prepared in 6.1.1 to a 25-mL volumetric flask. Make to volume with MeOH and mix well.

6.2 Calibration Blank (Standard 0)

Accurately pipette 0.25 mL of the ISS solution (prepared in 6.1.2) into a 25-mL volumetric flask.

Make to volume with MeOH and mix well.

6.3 Calibration Standards – Nicotine

6.3.1 1° Stock in MeOH (approx. 50 mg/mL)

Accurately weigh approximately 1.0 g nicotine, into a 20-mL volumetric flask.

Dissolve in MeOH then bring to volume and mix well.

6.3.2 2° Stock in MeOH

Transfer 1.0 mL of the nicotine 1° (*prepared in 6.3.1*) to a single 50-mL volumetric flask.

Dilute to volume with MeOH and mix well.

6.3.3 Calibration Standards

Take appropriate aliquots of the 2° stock *(prepared in 6.3.2)* and transfer to separate 25-mL volumetric flasks (Table 1).

Add 250 µL ISTD working solution (*prepared in 6.1.2*) to each.

Make to volume with MeOH and mix well.

Table 1. Nicotine calibration standards – Nominal Concentrations (actual concentrations will vary depending upon the amount weighed and the purity of the analyte)

Standard ID	Stock Vol. (mL)	Final Vol. (mL)	Nominal NIC Conc. (µg/mL)
1	0.1	25	4
2	0.2	25	8
3	0.5	25	20
4	1	25	40
5	3	25	120
6	5	25	200
7	10	25	400

7. PROCEDURE

7.1 Sample Extraction

7.1.1 Accurately weigh 0.25 ± 0.05 g of tobacco into a 50-mL PP centrifuge tube, or equivalent.

7.1.2 Add 4 mL NaOH pre-treatment solution.

7.1.3 Let samples sit for approximately 30 minutes.

7.1.4 Accurately add 400 µL ISSS and 40 mL MeOH.

7.1.5 Shake or stir for approximately 30 minutes.

7.1.6 Filter an aliquot into each of two autosampler vials.

Note: Adjust volumes as needed for portioned tobacco products. The ratio of ISSS to MeOH must be constant in all samples.

7.2 Sample Run Order

7.2.1 Priming Sample/System Suitability

A priming sample (matrix) must be injected from 2 to 3 times to ensure that active sites in the system are minimized.

7.2.2 Calibration Blank

At least one reagent blank should be injected to ensure no carryover from the sample.

7.2.3 Calibration Standards

Arranged in increasing levels of concentration, beginning and ending with a reagent blank.

7.2.4 Samples

Samples (including any blanks, recoveries and/or reference samples) are typically analyzed in batches of ten to twelve or less.

7.2.5 Continuing Calibration (CC)

CC standards are analyzed to verify that the calibration is still valid. A reagent blank should be analyzed after every CC to monitor the carryover.

7.3 GC-MS Apparatus and Operation Parameters

The MS is operated in EI/SIM mode. The retention times and ions monitored are listed in Table 3.

Table 2. GC-MS condition and parameters.

Analytical Column	CAM column (30m x 0.25mm id x 0.25µm df) or equivalent polar, base-deactivated PEG column (e.g. Stabilwax-DB or Carbowax Amine)
Column Flow	1.0 mL/min (UHP Helium)
Injection Port Temperature	230°C
Injection Port Liner	Ultra Inert split, straight with glass wool, or equivalent
Injection Volume	1 µL
Injection Mode	Split, 60:1
Initial Oven temperature	110 °C hold for 1 minute
Temperature ramp	10 °C/min to 235°C, hold 4.5 minutes or more
Transfer Line temperature	230°C
MS Source temperature	230°C
MS Quad temperature	150°C
Solvent Delay	Approx. 5 minutes

Table 3. Analyte retention times and ions monitored.

Name	MW	Ret Time (min.)	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Internal Standard
Quinoline	129.2	8.9	129	N/A	N/A
(-)-Nicotine	162.2	8.2	84 or 162	162 or 84	Quinoline

7.4 Integration/Quantitation Parameters

The relative areas of the analytes and the internal standard in each calibration standard are used to create a calibration curve. The curves are linear with 1/x weighting.

8. CHROMATOGRAMS

Figures 1-2 illustrate typical chromatograms that can be expected to be obtained with this method.

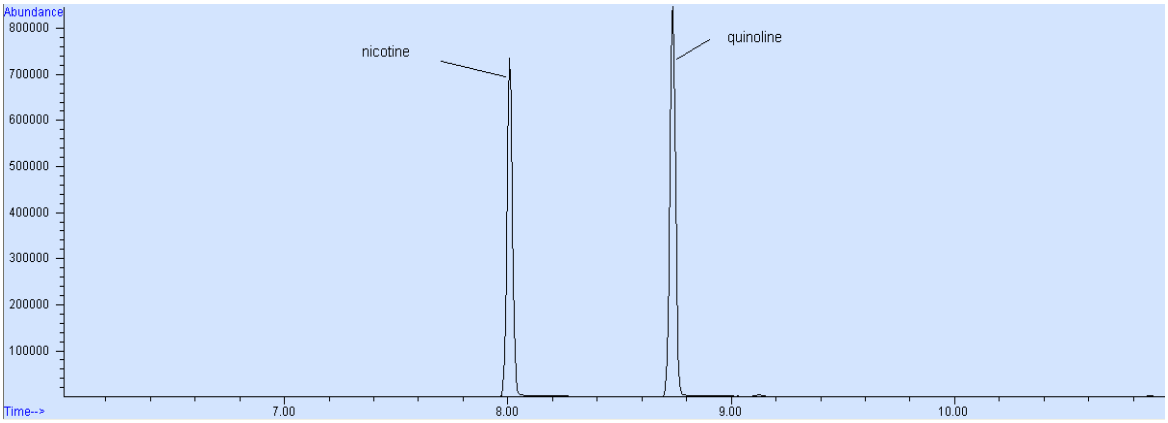


Figure 1. Total Ion Chromatogram for nicotine and quinoline in calibration standard (Nic ~ 100 µg/mL)

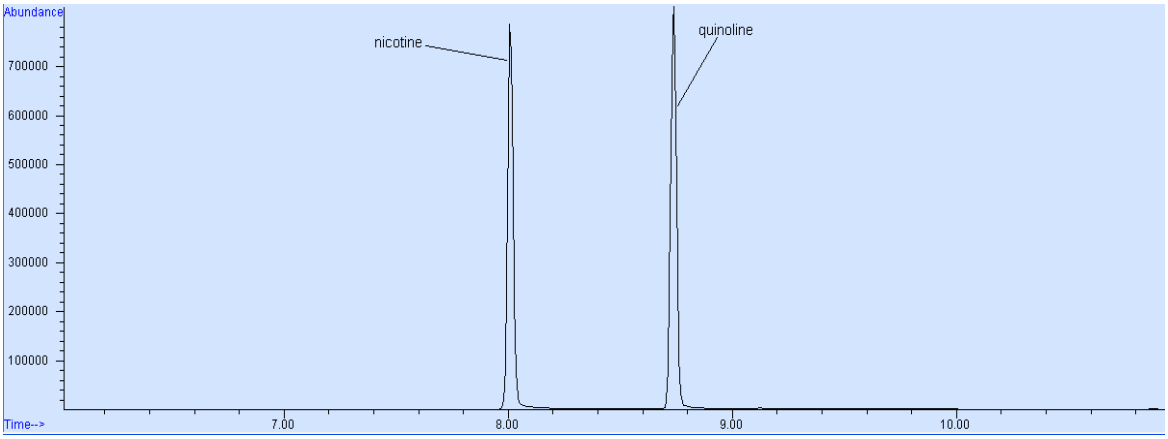


Figure 2. Total Ion Chromatogram for nicotine and quinoline in 3R4F sample extract.

9. CALCULATIONS

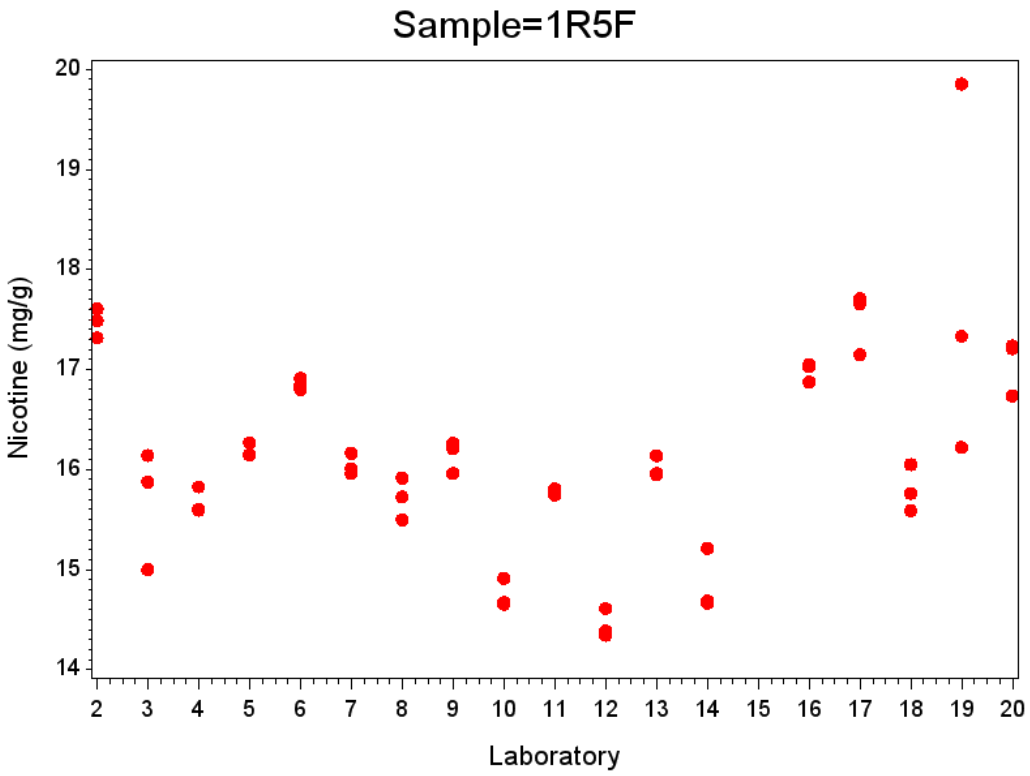
The analyte concentration (in µg/mL) is determined by the internal standard calibration method using the regression equation derived from the calibration curve. Results are then converted and reported on a per weight basis, typically µg/g.

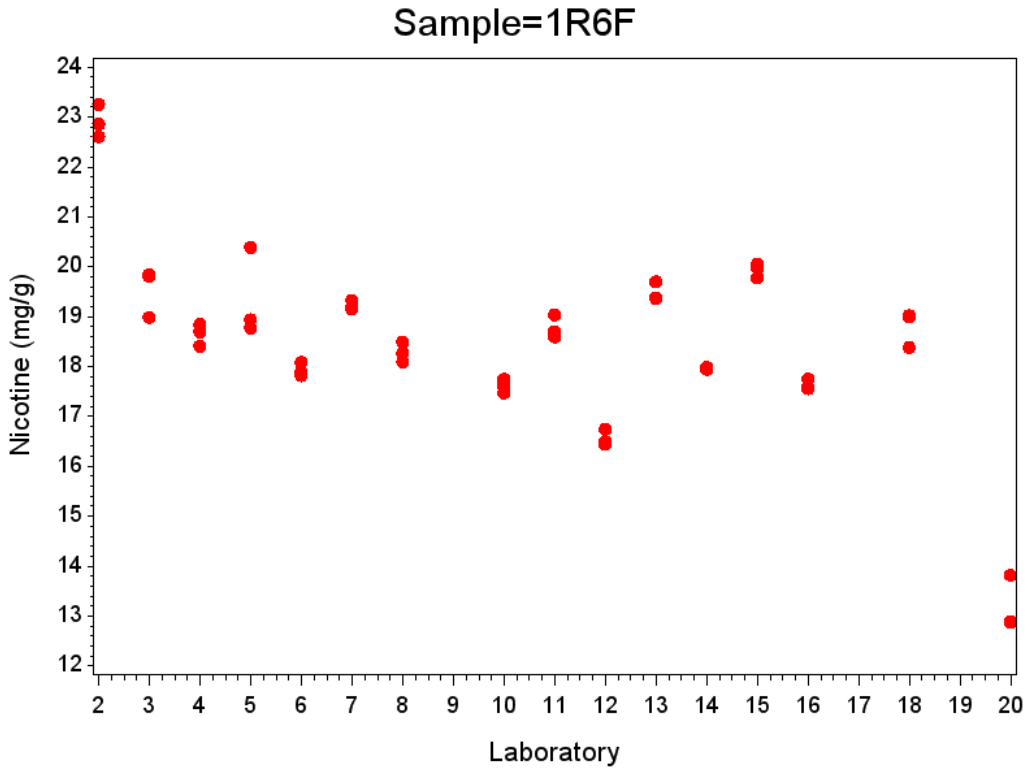
$$Analyte\ conc.(\mu g/g) = \frac{Analyte\ conc.(\mu g/mL) \times Sample\ Vol.(40\ mL) \times MF}{Sample\ Weight\ (g)}$$

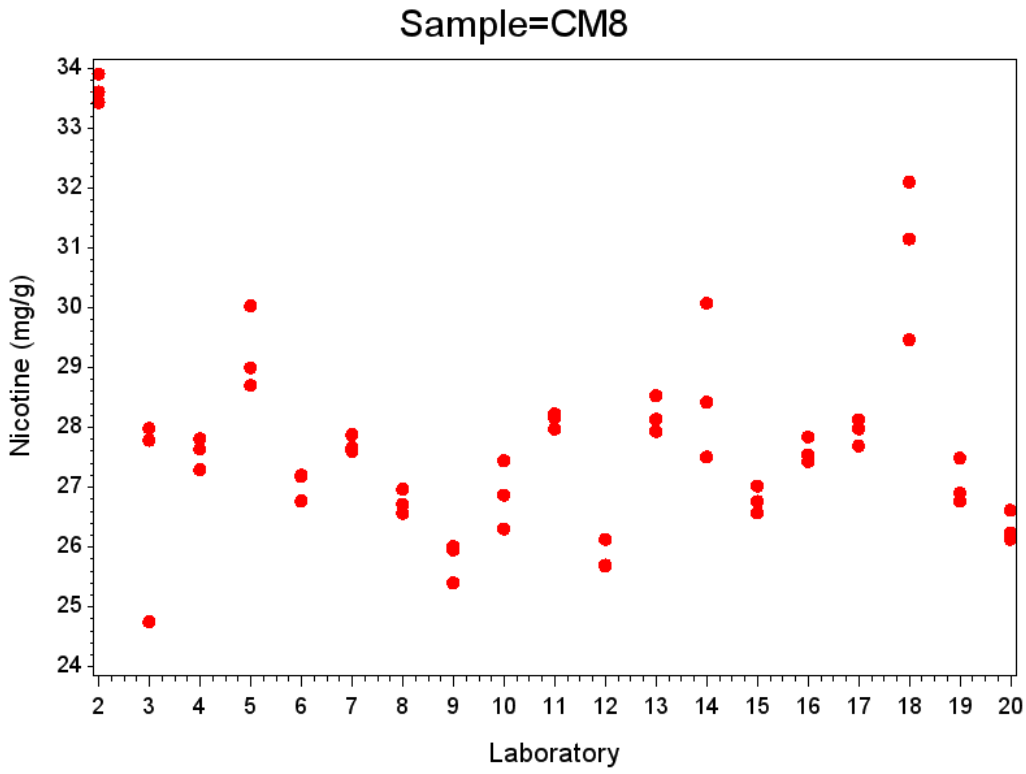
Note: there is no volume correction required for the addition of the pre-treatment solution as it dilutes all analytes and internal standards equally.
When reporting results on an 'as is' basis (i.e. not corrected for moisture content), the moisture factor (MF) value is 1. In instances where results are required to be reported on a dry weight basis (dwb), the following calculation is required to obtain MF for a sample:

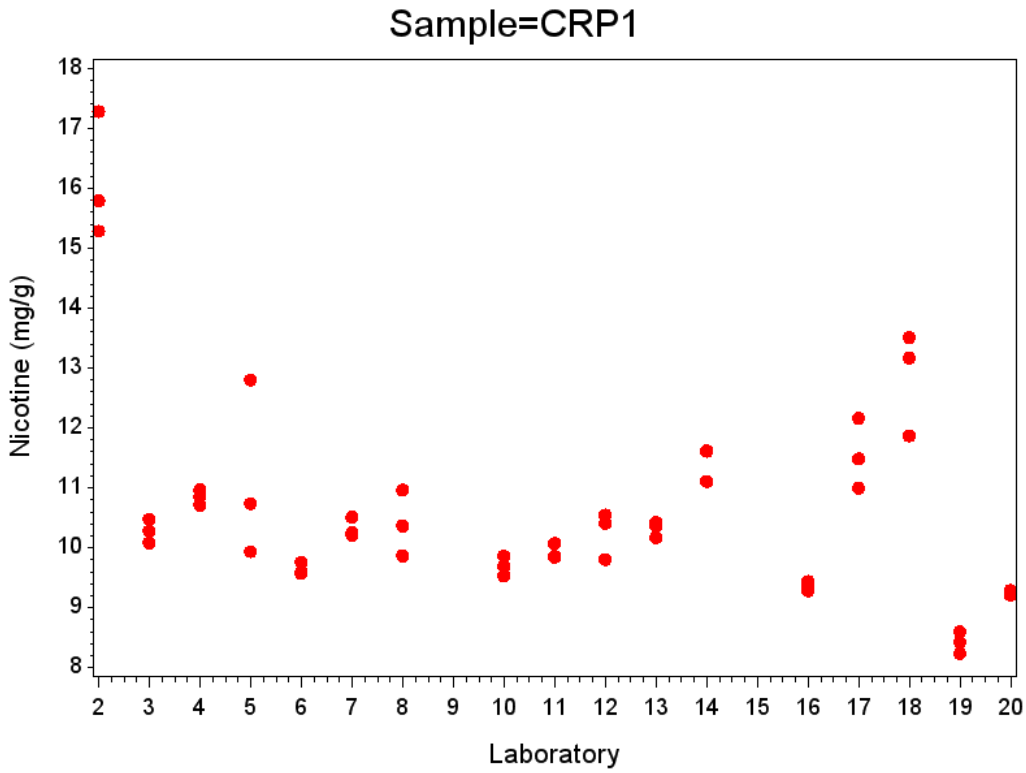
$$Moisture\ Factor\ (MF) = \frac{100}{100 - Moisture(\%)}$$

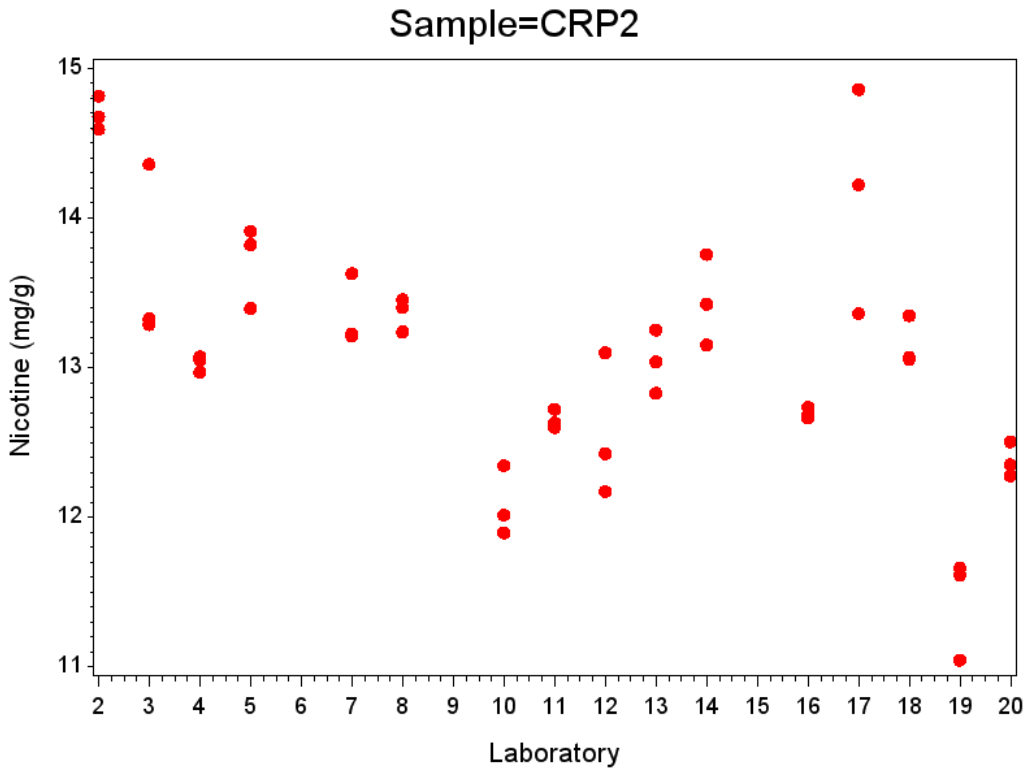
APPENDIX C: Raw Data Plots

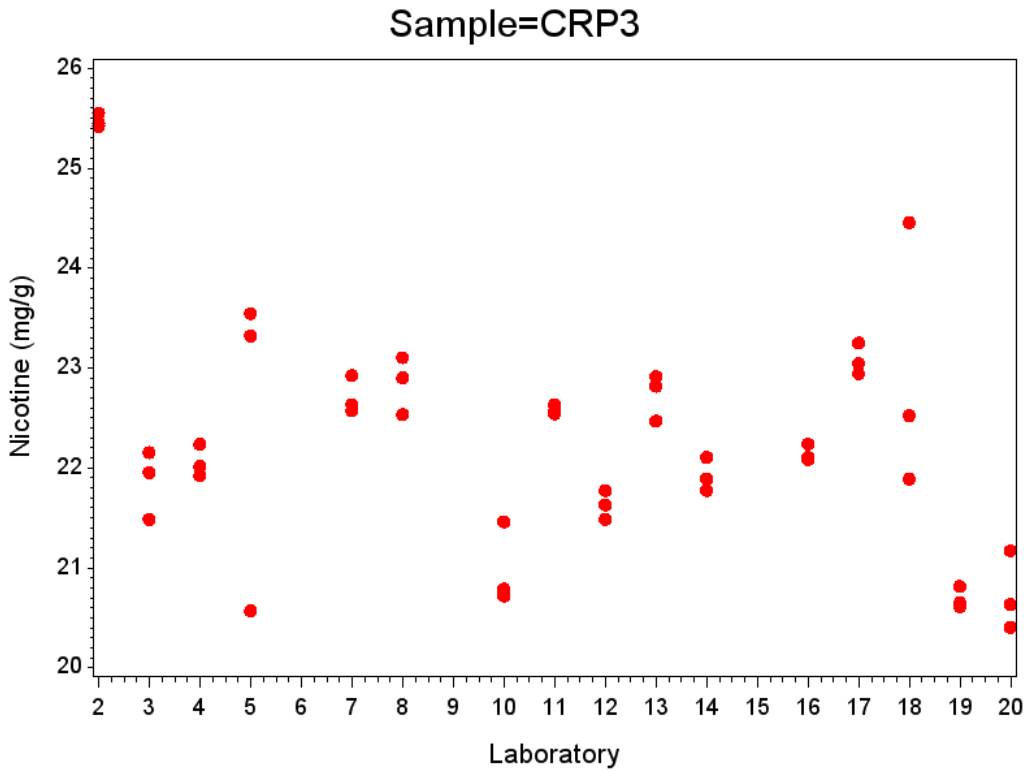


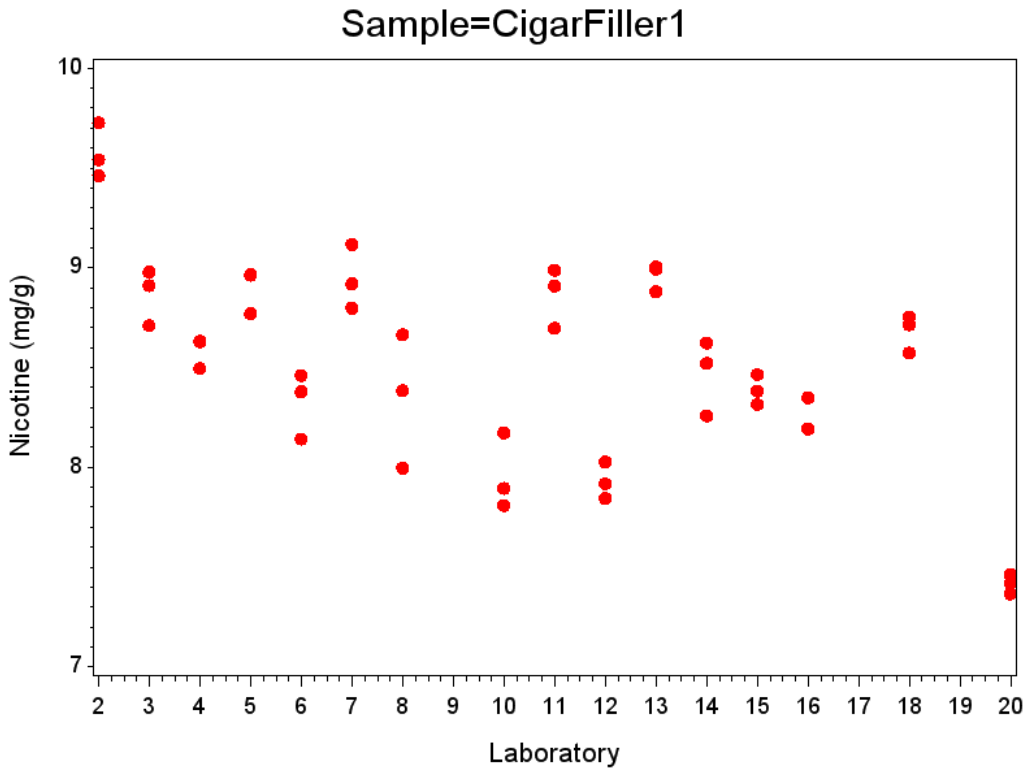


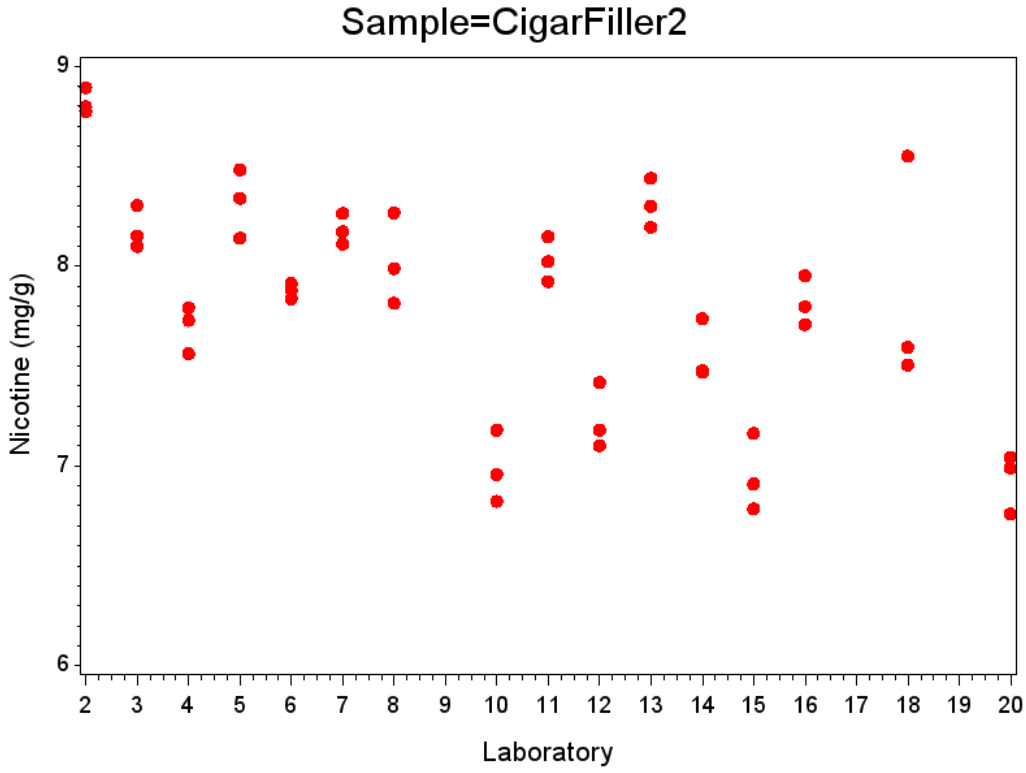

Sample=CM8
Nicotine (mg/g)
Laboratory

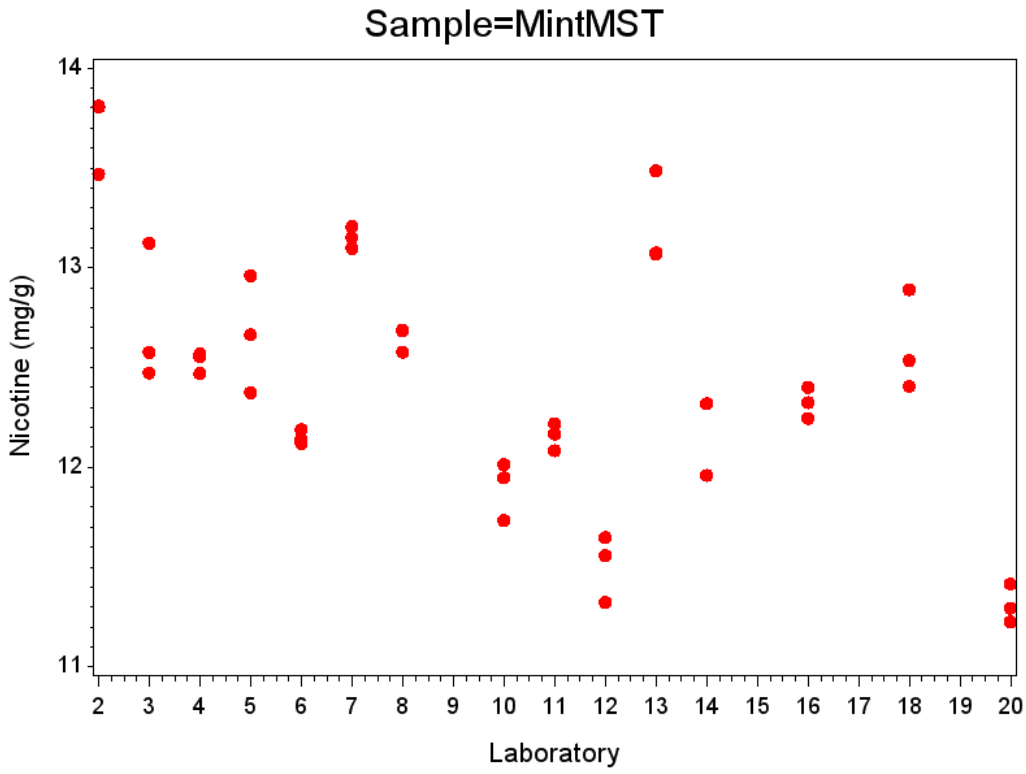

Sample=CRP1
Nicotine (mg/g)
Laboratory


Sample=CRP2
Nicotine (mg/g)
15
14
13
12
11
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=CRP3
Nicotine (mg/g)
26
25
24
23
22
21
20
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=CigarFiller1
Nicotine (mg/g)
10
9
8
7
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=CigarFiller2
Nicotine (mg/g)
9
8
7
6
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory


Sample=MintMST
Nicotine (mg/g)
14
13
12
11
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Laboratory

Trends in Plant Science

CellPress REVIEWS

Opinion

Challenges towards Revitalizing Hemp: A Multifaceted Crop

Craig Schluttenhofer[1,2] and Ling Yuan[1,2,3,*]

Hemp has been an important crop throughout human history for food, fiber, and medicine. Despite significant progress made by the international research community, the basic biology of hemp plants remains insufficiently understood. Clear objectives are needed to guide future research. As a semi-domesticated plant, hemp has many desirable traits that require improvement, including eliminating seed shattering, enhancing the quantity and quality of stem fiber, and increasing the accumulation of phytocannabinoids. Methods to manipulate the sex of hemp plants will also be important for optimizing yields of seed, fiber, and cannabinoids. Currently, research into trait improvement is hindered by the lack of molecular techniques adapted to hemp. Here we review how addressing these limitations will help advance our knowledge of plant biology and enable us to fully domesticate and maximize the agronomic potential of this promising crop.

Hemp: A Multifaceted and Diverse Plant

The genus *Cannabis* (commonly classified into the species *Cannabis indica*, *Cannabis sativa*, and *Cannabis ruderalis*) has been used for food, fiber, and medicine for over six millennia [1,2]. Depending upon the use, Cannabis is organized into two distinct groups – marijuana and hemp. Marijuana, primarily used recreationally for its intoxicating properties, may have medicinal value [3–5]. In contrast, hemp is valued for its medicinal compounds, fiber, and seed that are collectively used in over 25 000 known products [6]. Compared to marijuana, the medicinal compounds of interest found in hemp are nonintoxicating, for example, cannabidiol (CBD). In European and North American countries, to be legally classified as hemp the crop may not contain more than 0.2% or 0.3% of the intoxicating compound Δ^9-tetrahydrocannabinol (THC), respectively. This level of THC in Cannabis is insufficient to induce intoxication. Differences in cultural practices of marijuana and hemp result in minor variations in morphologies, allowing some distinction between these two crops [6].

Traditionally, hemp is grown for either seed or fiber. Hemp seeds contain approximately 30% protein, 25% starch, and 30% oil [7,8]. Pressed seeds release an oil that contains >90% polyunsaturated fatty acids. With a desirable ratio of ω-6 to ω-3 lipids [7,8], hemp seed oil is a valuable addition to human and animal diets [9]. Additionally, the oil can be used for cooking or processed into cosmetics and fuels [10,11]. The residual seed cake can be used for protein rich animal feed. **Bast fibers** (see Glossary) are primarily used to make high quality papers, whereas most **hurd** goes into animal beddings [10]. Recent technological advances have expanded the use of hemp fiber and hurd to include the production of carbon nanosheets, plastics, 3D-printer filaments, oil absorbent materials, and construction concrete. Additionally, hemp produces over 100 known cannabinoids, most notably CBD [12]. In the USA, clinical

Trends

For states which define hemp (<0.3% THC) as distinct from marijuana, the USA Agriculture Act of 2014 allows departments of agriculture or universities to cultivate hemp as part of a research pilot program.

As of 2017, at least 39 US universities and dozens of researchers have begun studying hemp, yet guidance on top research priorities are lacking.

While traditionally a fiber and grain crop, hemp has emerged as a source of nonhallucinogenic medicinal phytocannabinoids (e.g., CBD) with distinct properties from marijuana. Dozens of clinical studies are now investigating anecdotal uses of CBD to treat various medical conditions.

The last several years have seen advancements in understanding Cannabis genetics through publications of a draft genome, transcriptome sequencing, quantitative trait mapping, and genetic comparisons between hemp and marijuana.

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[2]The Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY 40546, USA
[3]South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China

*Correspondence: lyuan3@uky.edu (L. Yuan).

trials are investigating CBD for treatment of 26 medical conditions[i] (Table 1). Furthermore, CBD has been granted **orphan drug status** for eleven conditions (Table 1).

Recently, hemp production has expanded beyond Eurasia and Canada to include three additional countries: Greece, Malawi, and the USA (Figure 1A). The rapid expansion in the USA may considerably impact the global hemp market. Several recent reviews have provided detailed information on the biochemistry, breeding, ecology, genetics, morphology, pathology, physiology, and production of Cannabis [13–23]. Despite recent progress, much remains to be

Table 1. Ongoing USA Clinical Trials and Orphan Medical Conditions for Which CBD Has Been Approved

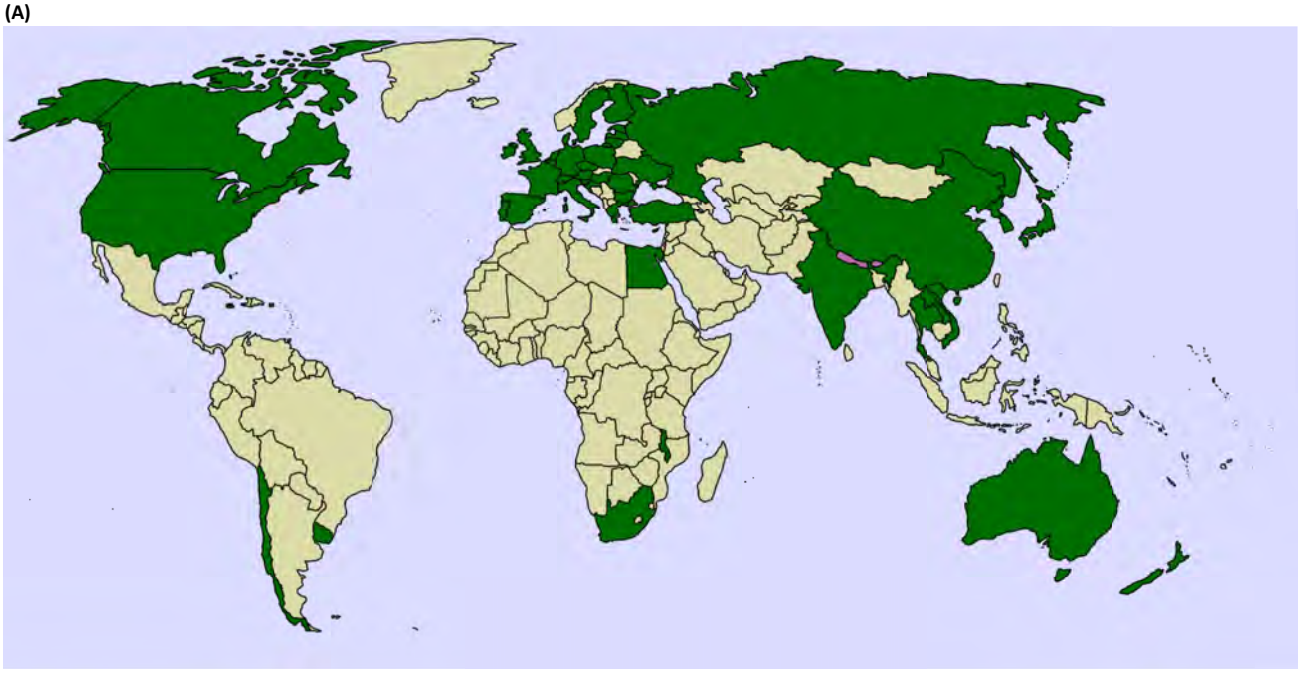
Medical condition[a]	Phase I	Phase II	Phase III	Orphan drug designation[b]
Amphetamine addiction	n.d.	Yes	n.d.	n.d.
Anxiety	n.d.	Yes	n.d.	n.d.
Autistic disorder	n.d.	Yes	n.d.	n.d.
Cannabis use disorder	n.d.	Yes	n.d.	n.d.
Cocaine dependence	n.d.	Yes	n.d.	n.d.
Dravet syndrome	n.d.	n.d.	Yes	Designated
Drug-resistant epilepsy	Yes	Yes	n.d.	n.d.
Epileptic encephalopathy	Yes	n.d.	n.d.	n.d.
Fatty liver	n.d.	Yes	n.d.	n.d.
Fragile X syndrome	n.d.	n.d.	n.d.	Designated
Glioblastoma multiforme	Yes	n.d.	n.d.	Designated
Glioma	n.d.	n.d.	n.d.	Designated
Graft versus host disease	Yes	Yes	n.d.	Designated
Graft versus host disease – prevention	n.d.	n.d.	n.d.	Designated
Infantile spasms	n.d.	Yes	Yes	Designated
Lennox–Gastaut syndrome	n.d.	n.d.	Yes	Designated
Neonatal hypoxic ischemic encephalopathy	n.d.	n.d.	n.d.	Designated
Lung cancer	Yes	n.d.	n.d.	n.d.
Opiate addiction	Yes	Yes	n.d.	n.d.
Pain	Yes	Yes	Yes	n.d.
Parkinson disease	n.d.	Yes	n.d.	n.d.
Pediatric intractable epilepsy	Yes	Yes	n.d.	n.d.
Pediatric schizophrenia	n.d.	n.d.	n.d.	Designated
Posttraumatic stress disorder	n.d.	Yes	n.d.	n.d.
Prader–Willi Syndrome	n.d.	Yes	n.d.	n.d.
Schizophrenia	Yes	Yes	n.d.	n.d.
Solid tumor	n.d.	Yes	n.d.	n.d.
Sturge–Weber Syndrome	Yes	Yes	n.d.	n.d.
Treatment-resistant seizures	Yes	n.d.	Yes	n.d.
Tuberous sclerosis complex	n.d.	n.d.	Yes	Designated
Ulcerative colitis	Yes	Yes	n.d.	n.d.

Abbreviations;: n.d., no data.
[a]Current as of February 16th, 2017 and considers CBD-only or high CBD/low THC formulations.
[b]Orphan drug designation.

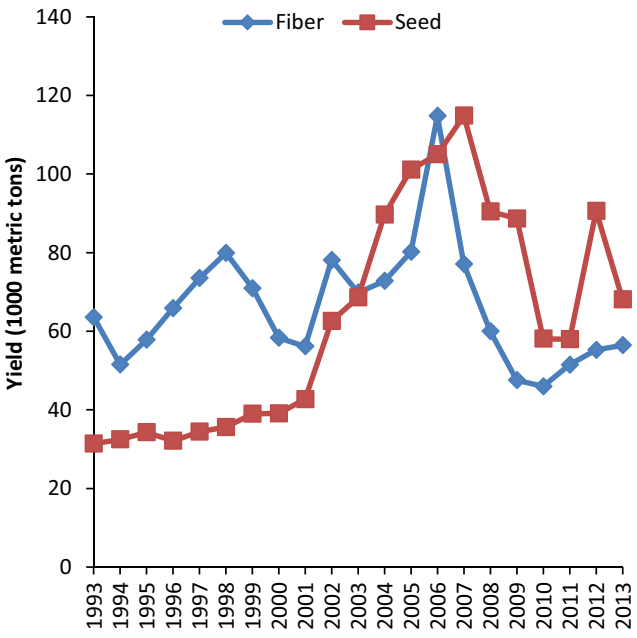
Glossary

Anemophilous: wind-pollinated.
Autoflowering: when flowering occurs independently of the photoperiod.
Bast fiber: long cellulose-rich bundles of phloem cells located underneath the stem epidermis.
Dioecious: male and female flowers are found on separate plants.
Female predominant: a population of consisting of 70–95% female plants and with the remaining plants being male or monoecious.
Hurd: the inner woody core of hemp stems.
Monoecious: separate male and female flowers are found on the same plant.
Orphan drug status: a special designation given by the USA Food and Drug Administration to a drug being investigated to treat a rare disease or disorder that affects less than 200 000 people in the USA, or for larger populations where investments are not expected to be recovered.
Shattering: the loss of mature seeds from the inflorescence.

(A)



(B)



(C)

Country	Fiber (ha)	Seed (ha)	Total ha†
Austria	600	–	600
Canada*	n/a	n/a	34262
Chile	4500	2200	6700
China	5000	5500	10500
Czech Republic	200	–	200
France	600	7706	8306
Hungary	300	1400	1700
Italy	425	–	425
Netherlands	1284	–	1284
North Korea	20000	–	20000
Poland	70	60	130
Romania	1600	1440	3040
Russia	4000	1644	5644
South Korea	12	–	12
Spain	10	2	12
Turkey	20	1	21
Ukraine	2000	2100	4100
Total	40621	22053	90906

Trends in Plant Science

Figure 1. Global Production of Hemp Fiber and Seeds. (A) Countries cultivating (green) or utilizing natural hemp populations for textiles (purple; Nepal and Bhutan). (B) Global production of hemp for fiber and seed from 1993 to 2013. (C) Countries producing hemp. Data was collected from the FAO for the year 2013. Total land area may be smaller depending on the quantity of dual-purpose (i.e., cultivars used for seed and fiber production) hemp planted. Annual statistics are from the FAO. Abbreviation: ha, hectares. *Value is from Health Canada for the year 2015. †Total production area assumes a single-purpose crop.

learned about this multifaceted and diverse plant. Importantly, there is limited information available about key research challenges that need addressed to improve this valuable crop. Thus, our objectives are (i) to briefly highlight the renewed interest in hemp, and (ii) illustrate strategic issues that should be addressed by researchers. While we focus on traits to improve hemp yield, these target research topics, in the long term, will also reveal important information about basic plant biology and domestication.

The Global Hemp Market

Viewed as an eco-friendly and highly sustainable crop [24], the global market for hemp is predicted to double from the year 2016 to 2020. At present, hemp is cultivated for commercial or research purposes in at least 47 countries, and it is utilized by indigenous populations for textiles in another two countries (Figure 1A). Since 2011, there has been an increase in hemp tonnage and acreage worldwide (Figures 1B and 1C). Statistics for hemp production are available from the Food and Agriculture Organization of the United Nations (FAO) for 16 countries (Figure 1C). Canada, China, Chile, France, and North Korea are currently the largest producers of hemp.

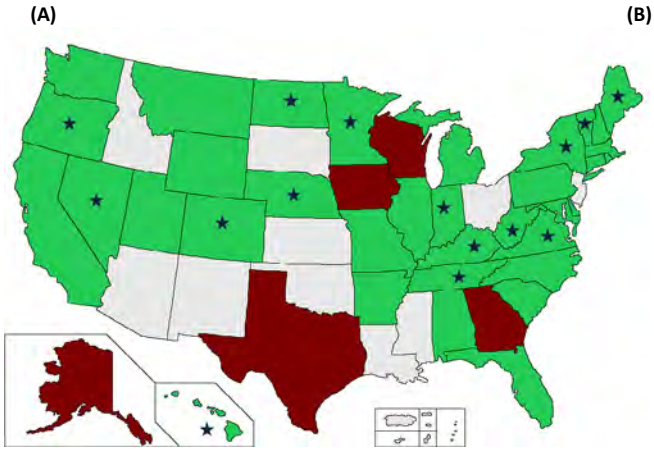
The USA is the largest importer of hemp products [6], obtaining most of its seed and fiber from Canada and China, respectively. In the Agricultural Act of 2014, the USA government authorized research into industrial hemp production (Figure 2A). Consequently, hemp production and research have rapidly increased in multiple states (Figure 2B). Establishment of a USA hemp industry may impact global commerce by reducing hemp imports from exporting countries.

As consumer demand for organic and environmentally sustainable products increases, there is a potential for significant growth of the world hemp market. Currently, there is a major discrepancy in crop value depending on product type; for example, the value of CBD far exceeds that of seed or fiber (Figure 2C). Using 2015 market prices and excluding costs [25], revenue/ha is estimated to range from $625–$25 000 (Figure 2C). To advance the industry, a focus on developing or improving products that can penetrate multibillion dollar markets (e.g., livestock health, improved construction materials, or energy storage) should be encouraged. Increasing demand for hemp-derived products will help solidify a long-term sustainable market.

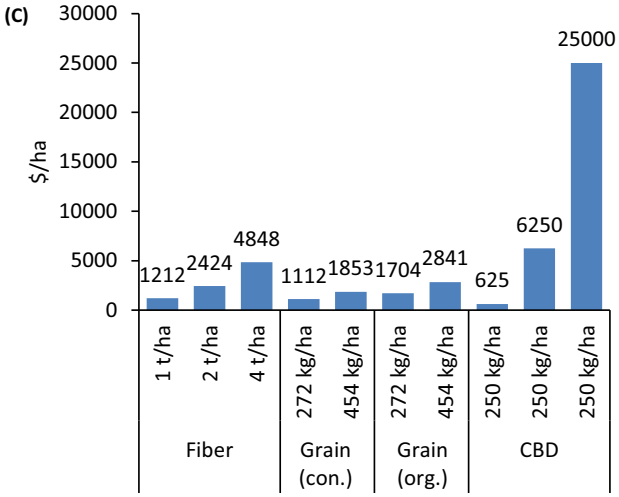
Future Directions for Hemp Research

Hemp is a genetically diverse and variable crop that produces raw products in three distinct categories: seed/oil, fiber, and metabolites. Within each category, hemp can be improved by multiple avenues of research. We highlight key research areas which increase grower yield or product quality for processors. These topics are not exhaustive, but are intended to guide research to areas which are of the highest priority.

Notably, due to the diverse nature of raw products produced from hemp, research targeting hemp yield traits will improve our understanding of basic plant biology. Seed and oil research will enrich our comprehension of grain yield and composition. Research into hemp fiber will enhance our knowledge of stem development and composition, genetic regulation of fiber traits, and biofuel production. Studies targeting metabolite yield will expand insights into both Cannabis-specific and shared plant chemistries, interaction with biotic stresses, and trichome development. Investigations into the plasticity of hemp's sexual phenotype will contribute to identifying mechanisms underlying plant sex determination. Importantly, unlike previously domesticated crops [26,27], selection for increased hemp yield provides a unique opportunity to study plant domestication for grain, fiber, and chemistry traits. With hemp, unlike most other crops, these valuable characteristics can be studied within a single species for which they are essential to sustainable and profitable production.



State	Year	Area planted (ha)	# Universities
Kentucky	2014	13.35	6
	2015	373.12	8
	2016	1021.83	10
Colorado	2015	930.78	1
	2016	2396.35	2
Hawaii	2015	0.10	1
	2016	0.00	1
Indiana	2015	1.62	1
	2016	0.81	1
Maine	2016	0.40	0
Minnesota	2016	20.64	1
Nebraska	2016	0.00	1
Nevada	2016	87.53	1
New York	2016	12.14	3
North Dakota	2015	0.08	1
	2016	28.33	1
Oregon	2015	20.23	1
	2016	202.34	1
Tennessee	2015	283.28	2
	2016	91.05	2
Vermont	2015	8.09	1
	2016	24.28	1
Virginia	2016	14.97	3
West Virginia	2016	4.05	1
Total	**2014**	**13.35**	**6**
Total	**2015**	**1617.31**	**16**
Total	**2016**	**3904.73**	**29**

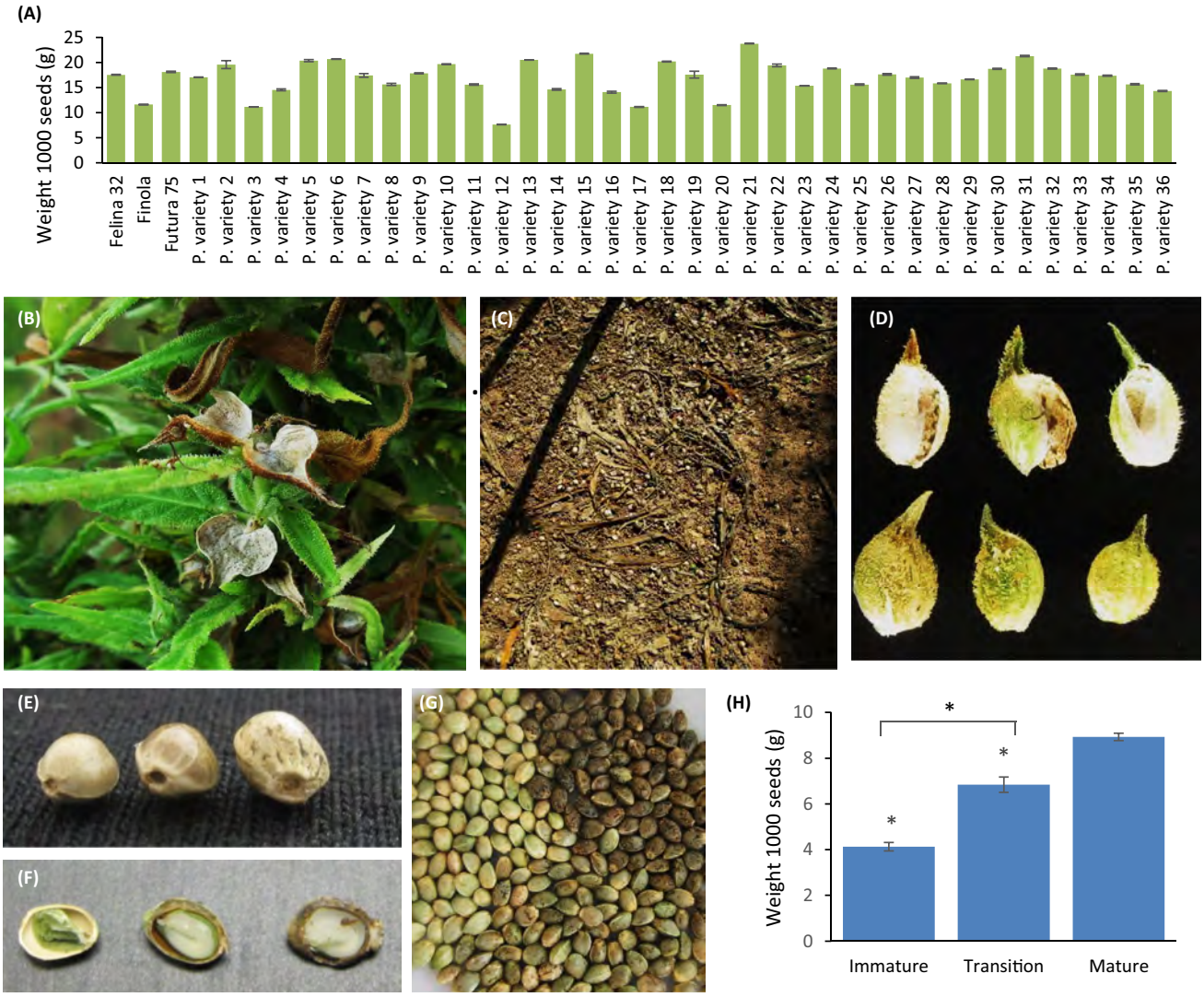


Trends in Plant Science

Figure 2. Hemp Production in the USA. (A) USA states currently able (green) and those pursuing legislation (red) to grow hemp, according to the 2014 Farm Bill. States that conducted hemp trials in 2016 are denoted with a blue star. In 2014, ten states (California, Colorado, Kentucky, Maine, Montana, North Dakota, Oregon, Vermont, Washington, and West Virginia) had in practice the necessary distinctions between marijuana and hemp, qualifying them for hemp research. Currently, 33 states meet the qualifying criteria. (B) USA states growing hemp, number of hectares planted, and the number of participating universities and colleges. Data obtained from VoteHemp[iv] and state Departments of Agriculture. (C) Revenue of hemp fiber, grain (conventional and organic), and phytocannabinoid (CBD) products per hectare. Prices are based on those paid during 2015. Fiber values assume a price of $1.21/kg of actual fiber. Assuming bast fibers make up 25% of the stem dry matter, then 1, 2, and 4 metric ton/ha are equal to 4, 8, and 16 metric ton/ha of stems, respectively. Grain production assumes a price of $1.65 and $2.54 per kilogram of conventional conventional (con.) or organic (org.) seed, respectively. Phytocannabinoid prices were obtained from hemp producers and CBD processors. CBD prices are complex, ranging from $2.50 to 10.00/g of pure CBD. Higher prices are paid for crops with higher percentages of CBD in flower material. Here, revenue per hectare was calculated using $2.50/g for 1%, $5.00 for 5%, and $10.00 for 10% CBD concentration in flower material.

Grain and Oil Production

As a semi-domesticated crop [28], many traits for hemp seed and oil yield require improvement; these include seed size consistency and improved **shattering** resistance. Significant advances in hemp seed production occurred with the development of FIN-314 ('Finola'), an **autoflowering** grain variety with a short stature, adaptation to high latitudes, and high yield [29], resulting in it presently being the most popular cultivar grown in Canada [30]. However, seed size is highly variable among hemp cultivars (Figure 3A) [16] and 'Finola' seeds are ~50% the size of many commercial varieties (Figure 3A). Selection for genetically stable cultivars with larger seeds will be important for increasing hemp grain yields.



(I)

Crop	Biomass† (Mg DM/ha/y)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Refs
Hemp fiber	7–34.0‡	73–77	7–9	2–6	[47–50]
Hemp hurd		34–48	21–25	17–19	[48]
Corn stover	4.6–5.5	38	26	17	[51,52]
Switchgrass	5.4–34.6	28–37	23–27	15–18	[53,54]
Giant *miscanthus*	10.0–44.0	50–52	25–26	12–13	[55]
Poplar	7.7–17.3	42–49	16–24	21–30	[56]
Willow	6.2–21.5	46–56	12–14	13–14	[57,58]

Trends in Plant Science

Figure 3. Properties of Hemp Seeds and Biomass. (A) 1000-seed weights for 39 hemp varieties. 'P. variety' designates a proprietary variety. (B) Seeds remain weakly attached to the plant but are susceptible to loss due to shattering. (C) Seeds shattered onto the ground prior to harvesting. (D) Seeds still partially (top) or completely (bottom) encased in bracts and perianth. (E) Hemp seeds showing the point of disarticulation at the base. (F) Dissected immature, transitional, and mature seeds (left to right) showing stages of seed fill. (G) Counterclockwise from upper left: immature, transitional, and mature hemp seeds. Seeds at each stage of

(See figure legend on the bottom of the next page.)

During domestication, hemp has retained little resistance to shattering [14]. However, hemp field trials have revealed that significant grain is lost due to shattering prior to and during harvesting as a result of inconsistent inflorescence maturity (Figures 3B and 3C), particularly if collected outside of the optimal harvest time windows. To mitigate this problem, growers harvest seeds at 70% maturity[ii] [31]. Hemp inflorescences are large multi-seeded heads in which each individual seed is partially surrounded by a bract, and an abscission zone connects the hull to the pedicle (Figures 3D and 3E). Selection for a stronger-walled abscission zone or the prevention of bracts releasing seeds (Figures 3D and 3E) are possible physiological traits to target to reduce hemp seed loss due to shattering. Furthermore, immature seeds are similar in size, but weighed only half that of mature seeds due to incomplete embryo development (Figures 3F, 3G and 3H). Without shattering, immature seeds would all fully mature, increasing yield by up to 15%. Thus, further domestication of non-shattering cultivars could greatly improve yield via a multifold mechanism, preventing harvesting loss and permitting all seeds to reach maturity.

Seed traits that expand market options will also be valuable. For example, there has been little research investigating the differences in hemp seed flavors. Taste tests in our lab identified varieties with weak to strong flavors of hazelnut (cv. 'Georgina') or walnut (cv. 'CRS-1'), as well as one (cv. 'Victoria') with a mild flavor. More work has been done on altering seed oil composition [32], although hemp seeds already possess valuable ω-3 characteristics [7]. Hemp seed oil is ~85% polyunsaturated fatty acids with 60% and 24% being ω-6 and ω-3 fats, respectively [7]. Further increases in ω-3 fatty acid levels might foster the favorability of hemp seed for human and animal dietary needs. Overall, different tastes and oil compositions would expand the use of hemp seed in human and animal food products.

Production and Quality of Hemp Fiber

Hemp stalks contain two key fractions, the bast fiber and hurd. To separate bast fibers from the inner hurd, the stalks must undergo a process called 'retting'. Retting relies on the diverse microbial populations in the environment to break down pectin and other components that bind the fibers to the hurd tissue [33,34]. Crop maturity at harvest, retting method, environmental conditions, as well as the nature of the bacterial and fungal populations, are factors that impact retting [35–37]. Harvesting the crop at initiation of flowering improves fiber yield, strength, and quality [33,38,39]. Continuing studies on the biodiversity, relationships, and functions of microbial communities will improve our understanding of the retting process [40–43] and augment the consistency of obtaining high quality products. Retting methods, primarily dew- and water-retting, pose drawbacks, including inconsistent fiber strength and quality, and polluted wastewater, respectively [34]. Development of varieties having bast fiber with higher cellulose content as well as lower pectin and lignin cross-linkages may decrease the retting requirements, thus improving fiber strength and quality while saving time and labor.

Hemp is a rapidly growing plant that tolerates high planting density [30,44–47], and may therefore be suitable as a viable biofuel crop. The total biomass of hemp per hectare is similar to other energy crops, including giant miscanthus (*Miscanthus* × *giganteus*), poplar (*Populus* sp.), switchgrass (*Panicum virgatum*), and willow (*Salix* sp.) (Figure 3I). However, hemp may provide a key advantage; its bast fibers contain 73–77% cellulose, 7–9% hemicelluloses, and 2–6% lignin, compared to 48%, 21–25%, and 17–19%, respectively, in the hurd [48–50]. Thus, the

development are similar in size. (H) 1000-seed weights of immature, transitional, and mature seeds of the variety Big Al Kentucky Plume. *, p-values of t-test <0.001. (I) Fiber yield and composition of hemp compared to other proposed biomass crops. Also see [47–58]. †Weight of dry matter (DM) includes moisture content at time of harvest. ‡Biomass is for fiber and hurd combined. Typically, stem material is 20–30% fiber.
For a Figure360 author presentation of Figure 3, see the figure online at http://dx.doi.org/10.1016/j.tplants.2017.08.004#mmc1

concentration of digestible cellulose and hemicellulose is higher in hemp fiber than in other energy crops (Figure 3I) [51–58]. In contrast, the cellulose and hemicellulose contents of hemp hurd are comparable to that in stems of giant miscanthus, poplar, switch grass, and willow. Importantly, 20–30% of the stem biomass in hemp consists of high cellulose fiber; thus, the ratio of digestible sugars to lignin is higher in hemp than in other similar-yielding biofuel crops. These traits make hemp an above-average energy crop for some biochemical-based biofuel production and greenhouse gas abatement applications [59,60]. Establishment of hemp as a biofuel crop would be beneficial to the industry by increasing demand for hurd and fiber.

Phytocannabinoids and Other Metabolites

Hemp produces a diverse array of nonintoxicating phytocannabinoids, terpenes, and phenolic compounds with potential pharmaceutical values as drugs or supplements [3,61,62]. The biosynthesis of terpenophenolic phytocannabinoids in Cannabis is well understood, albeit, several early steps in the pathway remain to be characterized [63,64]. Understanding the regulation of phytocannabinoid biosynthesis is vital to development of varieties that are optimized for production of desirable metabolites while maintaining low levels of THC. Little is known about the endogenous and environmental regulation of phytocannabinoids. Abscisic acid, ethylene, and gibberellic acid modulate the production of phytocannabinoids [65–67]. However; at present, factors controlling the epigenetic, transcriptional, and post-transcriptional regulation of phytocannabinoid biosynthesis remain uncharacterized.

Hemp trichomes are classified into bulbous, capitate-sessile, capitate-stalked, and nonglandular types [13,68]. Phytocannabinoid production and accumulation are localized to the capitate-stalked glandular trichomes [13,69]. Increased production of phytocannabinoids in marijuana is, at least partially, due to the presence of larger glandular trichomes [70]. Elucidating hormonal and other signaling cascades that regulate the development and size of specific types of hemp trichomes will also be important in maximizing phytocannabinoid production in hemp.

The effects of agronomic practices and nutrients on phytocannabinoid production also need to be investigated. Anecdotal claims from marijuana growers suggest that pollination of Cannabis flowers lowers phytocannabinoid yield [71], consistent with decreases in essential oil levels [72]. Further studies to evaluate this concern are essential to maximize the production of CBD and other desired phytocannabinoids.

Hemp Breeding Limitations

Germplasm collections are a fundamental source of genetic and phenotypic diversity for plant breeding and research. Currently, access to and utility of accession collections remain limited due to the lack of a core Cannabis germplasm collection. As THC levels may limit germplasm utility in many regions, accessions with $<0.3\%$ THC should be used to form a hemp-only germplasm core collection. Establishment of a core collection encompassing the range of hemp genetic and phenotypic diversity would increase the utility of germplasm resources and be invaluable for breeding and genetic analyses. Comparisons of accessions present in existing collections [73] are needed to help establish such a core collection. Similarly, centralized and curated collections of hemp mutants are not available. The development of mutant germplasm collections will provide a rich source of genetic variation for studying gene function and improved traits for breeding.

Hemp is an **anemophilous** crop in which the pollen can travel long distances. Studies in southern Spain identified Cannabis pollen in atmospheric samples which arrived from the extensive marijuana fields in Morocco over 100 km away [74,75]. Long-distance pollen dispersal causes difficulty for breeding programs which require spatial or mechanical methods for

germplasm isolation. Cost-effective and efficient methods are needed that will allow breeders to develop multiple new hemp varieties simultaneously in a limited growing area.

Hemp Sex Expression

Hemp is a **dioecious** plant with female and male hemp plants being valued differently depending upon the products. For phytocannabinoid production, a pure female population is most desirable. As a seed crop, a **female predominant** population, with a limited number of male plants for pollination, or a **monoecious** variety, is most desirable to maximize yield. For fiber production, males and females are both utilized, although males are preferred [15,18]. Therefore, a major goal of hemp growers and breeders is to quickly and easily determine or manipulate the sex of plants, preferably prior to planting.

Sex in hemp is genetically determined by a pair of heteromorphic sex chromosomes; females have an XX chromosome pair whereas males have XY. However, environmental conditions (e.g., photoperiod and temperature) and phytohormones can affect sexual phenotype [15,76–79], suggesting other overriding regulatory mechanisms are involved in determining sex in hemp. Monoecious cultivars possess XX sex chromosomes [80], but they produce flower clusters with male flowers at the bottom and females towards the top of each inflorescence [14]. Notably, male flowers occur as the plant transitions from rapid growth to flowering. Stem elongation and fiber development are associated with elevated levels of gibberellins [81]. In hemp, gibberellins are associated with plant masculinity and greater fiber number, length, and diameter [77,82,83]. Thus, a concentration gradient of gibberellin and other hormones may dictate inflorescence sex.

Genetic markers have been developed to differentiate sex in hemp plants [84–86]; however, such a method is not viable for commercial plantings. Recently, quantitative trait loci (QTLs) were identified for sex expression in dioecious and monoecious hemp [87,88]. Cloning of the responsible genes from these QTLs will greatly improve our understanding of genetic control of sex in hemp. Identification of genes present on the sex chromosomes, especially outside the pseudoautosomal recombinant region [86], will be critical for understanding sex-linked traits. Continued development of molecular markers is needed to improve QTL mapping resolution and for marker-assisted selection of desirable traits in breeding programs.

Hemp Molecular Biology

The organic food market is a key player in promoting hemp food and CBD products. As such, widespread public acceptance of transgenic hemp is unlikely. It also remains unknown whether the public will welcome hemp modified using gene-editing techniques, which lack nonplant or plant-pest DNA sequences [89,90]. Thus, many improvements to hemp will probably be accomplished using traditional breeding methods. However; for research purposes, the development of applicable molecular biology techniques is imperative to further study the molecular mechanisms that determine important traits in hemp.

Publication of a draft-quality Cannabis genome and other genetic studies have shed some light into the difference between marijuana and hemp. The Cannabis draft genomeAppendix A[iii] has been compared with draft genome sequences of its closest relative common hop (*Humulus lupulus*; Cannabaceae) as well as more distant species including breadnut (*Artocarpus camansi*; Moraceae) and mulberry (*Morus notabilis*; Moraceae) [91–94]. Recently, low coverage (4–6X) whole-genome sequencing and genotyping-by-sequencing have been performed on 54 (11 hemp and 43 marijuana) and 325 (55 hemp and 213 marijuana) distinct cultivars, respectively [94–97]. However; with only raw data files available, the lack of websites with easy-to-use graphical user interfaces for data analyses limits the utility of these draft-level genome sequences. Transcriptome assemblies are also available (Medicinal Plants Genomes Resource

and PhytoMetaSyn databases), but are primarily targeted toward understanding phytocannabinoid metabolism. Comparison of marijuana and hemp indicates that the expression of phytocannabinoid biosynthetic genes is higher in marijuana, suggesting that transcriptional regulation of the pathway may be one factor controlling cannabinoid production [94]. Recently, a transcriptome was generated for hemp bast fibers at different growth stages [98], providing insight into fiber development. The evolution of genetic differences between seed/oil, fiber, and dual-purpose cultivars is less studied. In-depth genetic comparisons of diverse seed/oil, fiber, and phytocannabinoid cultivars are needed to identify the specific genes and mechanisms controlling important yield traits. To attain the full benefit of these and other studies across species, the genome sequence needs to be improved beyond draft quality, and websites with user-friendly graphical user interfaces must be developed.

To characterize hemp gene functions, methods to manipulate gene expression (e.g., via gene knockout or overexpression) are urgently needed. Protocols for developing transformed hairy roots and cell suspension cultures are available [99,100], but the utility of both methods is limited since neither tissue produces seed, fiber, or phytocannabinoids. A whole-plant regeneration protocol has been developed for marijuana [101], suggesting that the development of transgenic hemp plants is feasible. Virus-induced gene silencing methods would also prove useful for studying gene function, but thus far have been unsuccessful [64]. Alternatively, isolation of mutants from chemical mutagenesis screens is possible [32], but extremely difficult due to the anemophilous and dioecious nature of hemp. Currently, exploitation of the natural genetic diversity present within hemp may be the most straightforward way to study gene functions.

Concluding Remarks

Hemp is an unusually diverse crop that can contribute to the seed/oil, fiber, and medicinal product markets. The global market for products derived from hemp is anticipated to double by 2020, largely due to growth in the USA market. The areas of developing seed shattering resistance; increasing seed size; selecting for grain flavors; understanding the microbial populations involved in retting; characterizing and enhancing the properties of hemp useful for biofuel applications; elucidating environmental, hormone, and nutritional impacts on production and accumulation of CBD and other valuable metabolites; establishment of a core hemp germplasm collection; identification of methods to specifically manipulate hemp sex expression as desired; and developing a high quality reference genome with user-friendly interface need further research to improve crop yield to maintain long-term sustainable production and economic viability. Many needed crop improvements can be achieved through traditional plant breeding. However, studies to elucidate the underlying biology of hemp seed, fiber, and metabolite production are lacking (see Outstanding Questions). Immediate establishment of molecular biology techniques is essential to hemp research. Improvements in hemp genomics will advance our understanding of key agronomic traits. While many scientific advances are needed to revitalize hemp production, we have illustrated target areas which we have identified as top research priorities.

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Outstanding Questions

The genetics of hemp grain yield traits remains poorly understood. What mechanisms control yield and quality traits (shattering resistance, sex determination, flavor, and oil composition)? To what extent do shattering, immature seeds, and nonoptimal sex impact grain yield? Answers to these questions are needed to maximize grain production. Both traditional and molecular genetic studies will be instrumental to better understand these processes.

Fiber production is hindered by inconsistent product quality, primarily due to problems with the retting process. How can consistent high-quality retting be attained? While recent efforts have started identifying microorganisms involved with retting, more work is needed to fully understand this key process. Identification of microbe species responsible will help guide development of methods and products, which should improve retting consistency and, in turn, maximize fiber yield and quality.

While the phytocannabinoid biosynthetic pathway is mostly known, the regulation and other mechanisms controlling metabolite quantity remain ambiguous. Specifically, phytocannabinoid yield is known to vary considerably between hemp cultivars and within different environments. Despite this, little is known about environmental impacts on hemp metabolism. How does the environment influence phytocannabinoid levels? What role do hemp specialized metabolites contribute to stress tolerance? Studies are needed to identify regulatory factors controlling phytocannabinoid production, particularly those connected with responses to stress.

Progress has been made to understand the differences between hemp and marijuana. However, much less remains known about the genetic differences between fiber and seed/oil hemp cultivars. What genetic changes separate fiber, seed/oil, or dual purpose hemp cultivars? Identification of such changes will aid breeding efforts selecting hemp for specific purposes.

Resources

[i]clinicaltrials.gov

[ii]omafra.gov.on.ca/english/crops/facts/00-067.htm

[iii]http://genome.ccbr.utoronto.ca/cgi-bin/hgGateway

[iv]votehemp.com/legislation.html

Supplemental Information

Supplemental information associated with this article can be found online at http://dx.doi.org/10.1016/j.tplants.2017.08.004.

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ARTICLE ADDENDUM

Cytokinin signaling promotes differential stability of type-B ARRs

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ABSTRACT
Cytokinins control key aspects of plant growth, including shoot and root meristem development and the timing of senescence of leaves and stems. Cytokinin perception triggers a 2-component signaling mechanism that ultimately leads to phosphorylation-dependent activation of a class of transcriptional regulators called type-B ARRs (RRBs). We have recently shown that the stability of the RRB family member ARR1 is increased in response to elevated cytokinin concentrations. In contrast, cytokinin decreases the stability of the closely related RRB member ARR2. The molecular mechanism governing the differential stability regulation of these 2 closely related RRBs remains unknown.

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In Arabidopsis, cytokinin signaling is initiated by the cytokinin-induced auto-phosphorylation of histidine kinase receptors (AHKs).[1,2] The phosphatidyl group is then transferred via histidine phosphotransfer proteins (AHPs) to response regulators (RRs) that are activated upon phosphorylation. RRs form 2 functionally antagonistic classes: the response promoting type-B RRs (RRBs) and the response inhibiting type-A RRs (RRAs). RRBs are transcriptional activators that regulate the expression of primary cytokinin-response genes, including *RRAs*. RRAs in turn function as negative feedback regulators of cytokinin signaling through an unknown mechanism.[1,2]

We have recently shown that the cellular pool of the Arabidopsis RRB member ARR1 is comprised of 2 subpools: a subpool with stable ARR1 that cannot be depleted even after prolonged treatments with protein synthesis inhibitors and an unstable ARR1 subpool that can be detected only after treatments with a proteasome inhibitor.[3] In addition, we found that the unstable ARR1 subpool is increased in response to cytokinin treatment, implying that the cytokinin signal inhibits the proteasome-dependent degradation of ARR1. Interestingly, *RRA* loss of function also caused an increase in ARR1 level, suggesting that the RRAs inhibit cytokinin responses by promoting ARR1 degradation.

Contrary to our results,[3] earlier reports showed that ARR1 is an unstable protein, and that its proteasome-dependent degradation is not regulated by cytokinins.[4-6] However, these studies analyzed a chimeric ARR1 version in which the protein was fused to a peptide tag for immunodetection, while our study was based on detection of endogenous ARR1. This suggests that ARR1 fusion proteins do not accurately reflect ARR1 stability control. If tagging of ARR1 altered its stability, then the proteasome targeting of other RRBs may also be impacted by the presence of peptide tags. To address this question, we analyzed the stability of ARR2, a less abundant member of the *ARR1/ARR2 RRB* subfamily.[7] In mesophyll protoplasts, a transiently expressed ARR2-hemagglutinin (ARR2-HA) tagged protein was shown to be unstable and its degradation was accelerated by cytokinin treatments.[12] To determine the stability of unmodified ARR2, we generated ARR2-specific antibodies against a synthetic peptide NGRSSRKRKEEEVDC using a previously described methodology.[3] Contrary to ARR1, which was detectable with specific antisera,[3] we were unable to visualize the endogenous ARR2 protein by immunoblotting analyses. Therefore, we generated transgenic plants expressing the full-length *ARR2* cDNA from the strong constitutive CaMV 35S promoter (Fig. 1). Prior to protein stability assays, we tested whether the overexpressed ARR2 is indeed functional. It has been shown previously that high-level expression of RRBs alters development and induces cytokinin hypersensitivity.[8-10] Indeed, the strong overexpressing lines were dwarfed, had shorter roots (Fig. 1A), increased anthocyanin content (4.00 ± 0.01, and 5.64 ± 0.01 fold increase [$n = 3$] compared to Col-0 for lines #2 and #3, respectively) and shorter hypocotyls when grown in darkness ($72 \pm 3\%$ and $76 \pm 2\%$ [$n = 25$] of Col-0 hypocotyl length for lines #2 and #3, respectively). These phenotypes are characteristic of plants treated with cytokinins.[8-10] Additionally, both lines were hypersensitive to cytokinin in a shoot induction response assay (Fig. 1B).

Analyses of the ARR2 overexpression lines revealed that ARR2 abundance is indeed cytokinin-regulated in a manner opposite to ARR1. Like ARR1,[3] the ARR2 steady-state level was not affected by prolonged treatment with the translation inhibitor cyclohexamide (Fig. 1C), suggesting that this ARR2 version is not actively targeted for proteolysis, in contrast to what was reported earlier.[12] ARR2 abundance was increased upon treatment with the proteasome inhibitor MG132 suggesting that, like ARR1,[3] a fraction of ARR2 is highly unstable and immediately degraded after synthesis (Fig. 1C). However, contrary to the cytokinin-stabilized ARR1, we observed a decrease in ARR2 abundance in response to cytokinin treatment (Fig. 1D). This decrease required 26S proteasome activity as it was blocked by MG132 (Fig. 1E). Thus, contrary to ARR1, ARR2 is destabilized by increased cytokinin action. This result was in agreement

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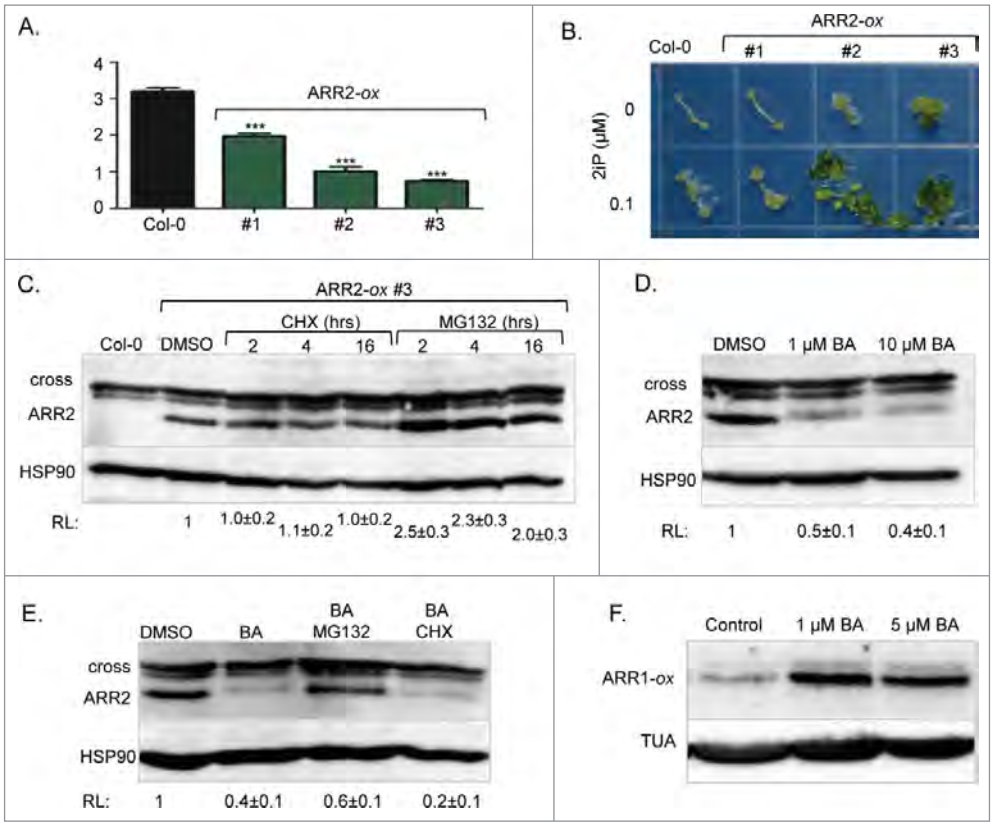

Figure 1. Stability control of ARR2. (A) Effect of ARR2 overexpression (ARR2-ox) on the root length. Seeds were sown and grown on vertically positioned plates with half-strength Murashige and Skoog growth (MS/2) media. After seven days of growth, seedlings were photographed and root length was measured using ImageJ. Data are presented and mean ± SD (n≥12). ***, p<0.001 (B) Shoot induction assay. Plants grown on MS/2 media for 7 d under dark conditions were exposed to light for 4 d to strengthen etiolated hypocotyls. Hypocotyls were then excised and placed on MS/2 media containing 1 μM NAA with or without 0.1 μM 2iP. Explants incubated on shoot induction media for 40 d were transferred to a new plate for photography. 2iP, N6-(2-isopentenyl)adenine. (C) Seven-day-old ARR2-*ox* #3 seedlings were treated for 2, 4 or 16 hours with 200 μM of the protein synthesis inhibitor cycloheximide (CHX) or 100 μM of the proteasome inhibitor MG132 as described.[3] Immunoblotting analyses were done as described.[3] Proteins were resolved on a 6% acrylamide SDS/PAGE gel. After probing with anti-ARR2 antibodies, membranes were re-probed with anti-HSP90 sera to demonstrate equal loading. Mean ± SD (n≥3) of the relative signal intensity (RL) is shown below the blot. (D) Seven-day-old ARR2-*ox* #3 seedlings were treated for 4 hours with 1 μM or 10 μM of the cytokinin BA. Immunoblotting was done as described in C. (E) Seven-day-old ARR2-*ox* #3 seedlings were treated for 4 hours with 1 μM of BA in the absence or presence of 100 μM MG132 or 200 μM CHX. (F) Seven-day-old transgenic plants overexpressing ARR1 (ARR1-*ox*) were treated for 4 hours with 1 μM or 5 μM BA. Proteins were resolved on 7.5% acrylamide SDS/PAGE gels. After probing with anti-ARR1 antibodies, membranes were re-probed with anti-α tubulin (TUA) antibodies.

with the earlier study that used ARR2-HA, suggesting that this fusion protein retained its cytokinin stability control.[12] However, the ARR1-HA version analyzed in the same study was shown to be cytokinin insensitive.[12] One possible explanation for this discrepancy is that ARR1-HA was expressed from a strong constitutive promoter and that this caused a deregulation of its cytokinin stability control. However, similar to its effect on endogenous ARR1, cytokinin treatment also increased the abundance of overexpressed and unmodified ARR1, indicating that increased expression of an RRB does not alter its cytokinin-regulated stability (Fig. 1F).

In conclusion, cytokinin-regulated proteolytic control of ARR1 and ARR2 differs; while cytokinin signaling led to accumulation of ARR1, it induced degradation of ARR2. This is unusual considering that in other hormone signaling pathways the perception of the signal led to either degradation or stabilization of functionally related pathway components.[11-13] In all described cases, a single E3 ubiquitin ligase or a family of specific E3s is responsible for signal-regulated protein degradation.[11-13] A previous study suggested that a family of F-box proteins targets ARR1 and ARR2 for proteasomal degradation.[5] However, this earlier report, which used chimeric RRB versions, did not reveal any effect of cytokinin on the stability of these 2 RRB members. This, in combination with our data, would suggest that there may be different RRB-targeting E3s whose actions are regulated by cytokinin. Indeed, cytokinin treatments lead to *de novo* synthesis of a battery of E3s, and their functions in the regulation of the cytokinin signaling pathway is still poorly understood.[14-16] Collectively, these results suggest that our current understanding of the molecular mechanisms that control RRB proteolysis is still incomplete.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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The miRNAome of *Catharanthus roseus*: identification, expression analysis, and potential roles of microRNAs in regulation of terpenoid indole alkaloid biosynthesis

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MicroRNAs (miRNAs) regulate numerous crucial biological processes in plants. However, information is limited on their involvement in the biosynthesis of specialized metabolites in plants, including *Catharanthus roseus* that produces a number of pharmaceutically valuable, bioactive terpenoid indole alkaloids (TIAs). Using small RNA-sequencing, we identified 181 conserved and 173 novel miRNAs (cro-miRNAs) in *C. roseus* seedlings. Genome-wide expression analysis revealed that a set of cro-miRNAs are differentially regulated in response to methyl jasmonate (MeJA). *In silico* target prediction identified 519 potential cro-miRNA targets that include several auxin response factors (ARFs). The presence of cleaved transcripts of miRNA-targeted *ARFs* in *C. roseus* cells was confirmed by Poly(A) Polymerase-Mediated Rapid Amplification of cDNA Ends (PPM-RACE). We showed that auxin (indole acetic acid, IAA) repressed the expression of key TIA pathway genes in *C. roseus* seedlings. Moreover, we demonstrated that a miRNA-regulated ARF, CrARF16, binds to the promoters of key TIA pathway genes and repress their expression. The *C. roseus* miRNAome reported here provides a comprehensive account of the cro-miRNA populations, as well as their abundance and expression profiles in response to MeJA. In addition, our findings underscore the importance of miRNAs in posttranscriptional control of the biosynthesis of specialized metabolites.

As sessile organisms, plants have evolved unique mechanisms to defend themselves in adverse environmental conditions. Plants synthesize thousands of specialized metabolites that play unique roles in plant growth, development, and defense. Many of these metabolites are beneficial for humans. *Catharanthus roseus* (L.) G. Don, commonly known as Madagascar periwinkle, synthesizes over 130 terpenoid indole alkaloids (TIA), including the pharmaceutically important anti-neoplastic compounds, vinblastine and vincristine[1]. Biosynthesis of TIAs is highly complex, involving multiple sub-cellular compartments. Tryptamine, derived from the indole branch, and secologanin, from the seco-iridoid branch, are condensed to form the first TIA, strictosidine, catalyzed by STRICTOSIDINE SYNTHASE (STR). The biosynthesis of TIAs is induced by a number of factors, including fungal elicitors[2], UV light[3], wounding[4], cold[5], and drought stress[6]. The phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are major elicitors of TIA biosynthesis in *C. roseus*. JA-responsive expression of TIA pathway genes are positively regulated by transcription factors (TFs), such as the JA-responsive AP2/ERFs, ORCA2[7], ORCA3[8], ORCA4 and ORCA5[9], as well as the bHLH factors, CrMYC2[10], BIS1[11], and BIS2[12], and the WRKY TF, CrWRKY1[13]. Several repressors, including the G-box binding basic leucine-zipper (bZIP)

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factors, GBF1 and GBF2[14], and zinc finger proteins, ZCT1, ZCT2, and ZCT3[15,16], negatively regulate the expression of TIA pathway genes in *C. roseus*. Other phytohormones, such as cytokinin (CK) and auxin, also affect TIA biosynthesis. In *C. roseus* cell suspension culture, CK enhances the accumulation of alkaloids[17], whereas auxin negatively regulates the expression of key TIA biosynthesis genes, including *TRYPTOPHAN DECARBOXYLASE* (*TDC*) and *STR*[18,19]. However, the molecular mechanism of auxin-mediated regulation of the TIA pathway is unknown. Auxin is an essential phytohormone that plays pivotal roles in plant growth and development. Auxin regulates gene expression through a signal transduction pathway which includes the F-box protein TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEINS (TIR1/AFB), AUXIN-RESPONSE FACTOR (ARF), and Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) proteins. ARFs are DNA binding proteins that recognize auxin-responsive (AuxRE) elements in target promoters to activate or repress expression[20]. In Arabidopsis, expression of *ARFs* are regulated posttranscriptionally by microRNAs (miRNAs)[21,22].

miRNAs comprise a major class of endogenous non-coding small regulatory RNAs approximately 21 to 24 nucleotides in length. They are present in a variety of organisms from algae to plants[23]. In plants, mature miRNAs are processed from primary transcripts by DICER-LIKE 1 RNase (DCL1), and subsequently loaded onto the ARGONAUTE (AGO) protein(s) to form RNA-induced silencing complexes (RISCs)[24]. The miRNA-loaded RISC binds to the target mRNA in a sequence-specific manner to either degrade the mRNA[25] or prevent it from translation[26]. Many plant miRNAs are evolutionarily conserved among plant species[27,28]. miRNAs are involved in numerous biological processes, including plant hormone homeostasis[29], root development[30,31], leaf morphogenesis[32,33], flower development[34], and embryogenesis[35,36]. However, information on the involvement of miRNA in regulation of specialized metabolite biosynthesis is limited[37–39]. Whether miRNAs are involved in regulating TIA biosynthesis in *C. roseus* has not been extensively investigated.

A previous study has identified 81 conserved and 7 novel miRNAs in *C. roseus* seedlings using deep sequencing[40]. The number of identified miRNAs in the report is considerably lower than the average number of miRNAs in plants studied thus far. Moreover, as the *C. roseus* genome sequence was unavailable during the previous study, the tomato genome was used as the reference to predict novel miRNAs. Here, we systematically identified miRNAs in *C. roseus* by sequencing four small RNA libraries from the control and JA-treated seedlings. We identified a total of 354 *C. roseus* miRNAs, including 181 conserved and 173 novel miRNAs. All identified miRNAs were mapped to the recently available *C. roseus* reference genome[41]. Expression of selected miRNAs and their potential targets were validated using quantitative reverse transcription PCR (qRT-PCR). In addition, we predicted a set of MeJA-responsive miRNAs that target a group of ARFs in *C. roseus*. Cleaved ARF transcripts in *C. roseus* cells were identified by Poly(A) Polymerase-Mediated Rapid Amplification of cDNA Ends (PPM-RACE). We hypothesized that ARFs bind to TIA pathway gene promoters to repress their activity, and at least some of these ARFs are regulated by miRNAs. We demonstrated that a JA-responsive, miRNA regulated ARF, CrARF16, binds to key TIA pathway gene promoters to repress their expression. Our findings provide a comprehensive account of the *C. roseus* miRNAome, and suggest that miRNAs and ARFs are involved in the regulation of TIA biosynthesis.

Results

Small RNA populations in *C. roseus* seedlings. A total of 12 small RNA (sRNA) libraries were constructed from the control and MeJA-treated *C. roseus* seedlings (Supplementary Fig. S1). Sequencing of small RNA libraries, from control and seedlings treated with MeJA for 1 h, 8 h, and 24 h, produced approximately 76.5 million (M), 76.8 M, 79 M, and 84.5 M raw reads, respectively (Supplementary Table S1). Among the raw reads, 88–91% were found to contain adapter sequences and thus chosen for further analysis. After removing low-quality reads and trimming the adaptor sequences, approximately 64.1 M, 65.5 M, 69.8 M, and 73 M clean reads were obtained for the control, 1 h-, 8 h-, and 24 h-MeJA-treated libraries, respectively (Supplementary Table S1). A total of 60.7 M (control), 62.2 M (1 h), 66.6 M (8 h), and 69.3 M (24 h) sequences were successfully mapped to the *C. roseus* reference genome. Sequences mapped to the *C. roseus* reference genome were further subjected to removal of transfer and ribosomal RNAs (t/rRNAs). Size distribution analysis of the sRNA sequences after filtering showed that all libraries exhibit similar distribution in length, with the most abundant class being 24 nt (Fig. 1A). After removal of redundant reads, the 24 nt reads remained most abundant, followed by the 23 nt reads (Fig. 1B). Overall, putative miRNAs and un-annotated sRNAs constitute 60–75% of the total cleaned reads in all twelve libraries (Fig. 1C).

Conserved miRNAs in *C. roseus*. To identify conserved miRNAs in *C. roseus*, the unique sequences were aligned to the mature sequences of known miRNAs deposited in the miRBase database[42]. A total of 181 conserved miRNAs, representing 33 miRNA families, were identified in *C. roseus* (Supplementary Table S2). Conserved miRNA varied from 18 nt to 24 nt in length. Most of the conserved miRNA families reported by Prakash *et al.*[40] are also present in our dataset. However, we could not detect miR169, miR530, miR828, miR2199, miR5139, miR5368, and miR6173 in our dataset; on the other hand, we detected miR2275, miR5532, miR5538, miR6300, miR6478, miR8016, miR858, miR397, miR390, miR393, miR394, and miR395, that were not reported by Prakash *et al.*[40]. In addition, 65 precursor sequences, representing 28 conserved miRNAs, were also predicted from the *C. roseus* genome (Supplementary Table S3). Several cro-miRNA families were found to be encoded by multiple loci in the *C. roseus* genome. For instance, cro-miR159 is encoded by two different loci (cro_scaffold_3041204 and cro_scaffold_3039079). Of the 65 identified precursor sequences, cro-miR159, -miR396, and -miR166 showed high abundance, having an average normalized count (NC) >1,000 (Supplementary Table S4), similar to those observed in other plant species, including radish and banana[43,44]. Cro-miR2275, -miR477, and -miR2111 were found to be least abundant, with an average NC less than 10. A considerable variation in expression was also noticed among loci coding for the same miRNA family (Supplementary Table S4). For instance, the average NC for the cro-miR319 family varied from 15–542. Similar variations in read abundance were also observed among members of the miRNA families such as cro-miR396 (1–1440), -miR395 (1–94), -miR171 (0.3–44), and -miR156

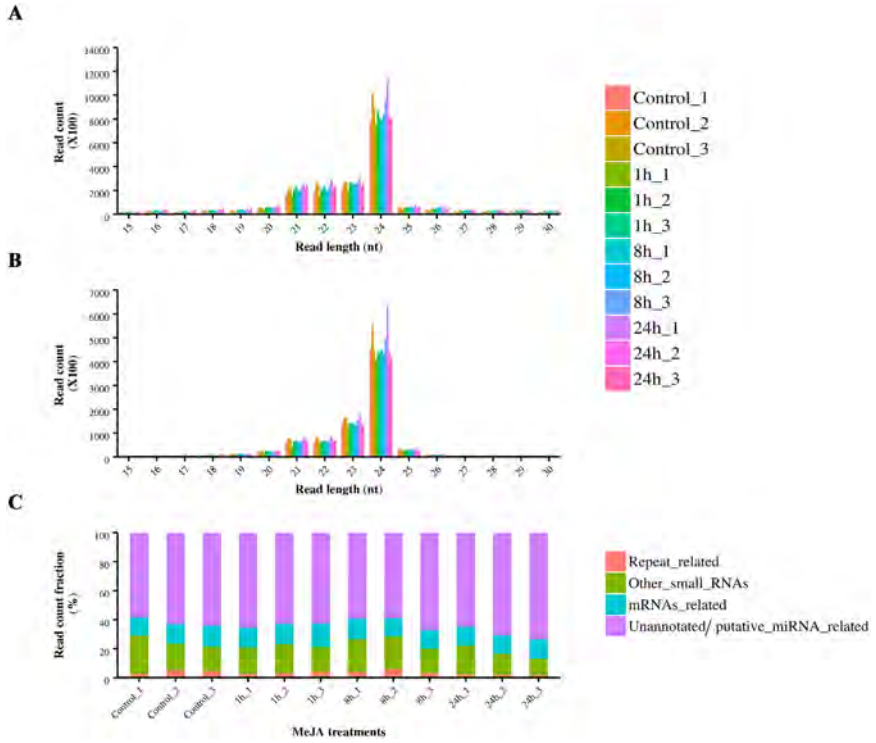

Figure 1. Length distribution and different classes of sequence reads identified in the small RNA libraries of *C. roseus*. **(A)** Size distribution of the total reads. **(B)** Size distribution of unique reads. **(C)** Distribution of different classes of sequence reads derived from the small RNA libraries. Putative miRNAs and un-annotated sRNAs constitute 60–75% of the total cleaned reads in all twelve libraries. Each small RNA library (control, 1 h-, 8 h-, or 24 h-MeJA treated) is represented by three biological replicates. *C. roseus* seedlings treated with 100 μM MeJA for 1 h, 8 h, or 24 h were used for RNA isolation and library preparation. Mock-treated seedlings served as control.

(1–194) (Supplementary Table S4). The average minimal folding free energy (MFE) value of the miRNA precursors was −49.2 kcal mol^{-1}; and the length of the precursors ranged from 75 nt to 377 nt, with an average length of 166 nt (Supplementary Table S3). The predicted secondary structures of the known miRNA precursors and locations of mature miRNAs in precursors were shown in Supplementary Fig. S2. A significant difference in the number of members was recorded for each conserved miRNA family (Fig. 2A). The largest family identified was cro-miR396 with 22 members, followed by cro-miR319 and -miR166, with 16 and 15 members, respectively. Of the remaining 30 families, 21 families were represented by 2–13 members while 9 were represented by a single member (Fig. 2A). Considerable variation in expression was observed among the individual families (Fig. 2B), and also among members of the same miRNA family. For instance, of the 6 members of the cro-miR159 family, cro-miR159a showed highest abundance (average NC > 1,000) while cro-miR159c was found to be least abundant (average NC < 1.0) (Supplementary Table S5).

Novel *C. roseus* miRNAs. A total of 173 novel *C. roseus* miRNAs were predicted based on established prediction criteria[45,46] (Supplementary Tables S6 and S7). The length of these novel miRNA precursors, ranging from 55–491 nt and averaging in 137 nt, agreed with the commonly observed length of miRNA precursors in plants[47]. The average minimal folding free energy (MFE) value of these miRNA precursors was −43.2 kcal mol^{-1}. The predicted secondary structures of these novel miRNA precursors and locations of mature miRNAs in precursors are shown in Supplementary Fig. S3. The sequence read abundance of the majority of *C. roseus* novel miRNAs were low compared to the conserved cro-miRNAs (Supplementary Table S8). A considerable variation in expression was also observed in *C. roseus*-specific miRNAs. Of the 173 *C. roseus*-specific miRNAs, the NC for 22 miRNAs were less than 10, whereas that for 134 miRNAs ranged from 10 to 200. Seventeen abundant novel *C. roseus*-specific miRNAs, including cro-novel-35, cro-novel-51, and cro-novel-56, have NC of more than 200 (Supplementary Table S8). The authenticity of predicted novel miRNAs is usually supported by the existence of complementary sequences[46]. In *C. roseus*, 162 of the 173 novel miRNAs have complementary miRNAs.

MeJA-responsive miRNAs in *C. roseus* seedlings. We analyzed the small RNA libraries to identify differentially expressed miRNAs (DEMs) in *C. roseus* seedlings with or without MeJA treatment. We identified 40 (14 upregulated and 26 downregulated), 71 (38 upregulated and 33 downregulated), and 47 (26 upregulated and 21 downregulated) JA-responsive miRNAs after 1 h, 8 h, and 24 h of MeJA treatment, respectively (Fig. 3A and Supplementary Table S9). Thirty-six conserved miRNAs, belonging to 14 miRNA families, such as cro-miR160 and -miR164, were differentially expressed in at least one time-point. Additionally, expression

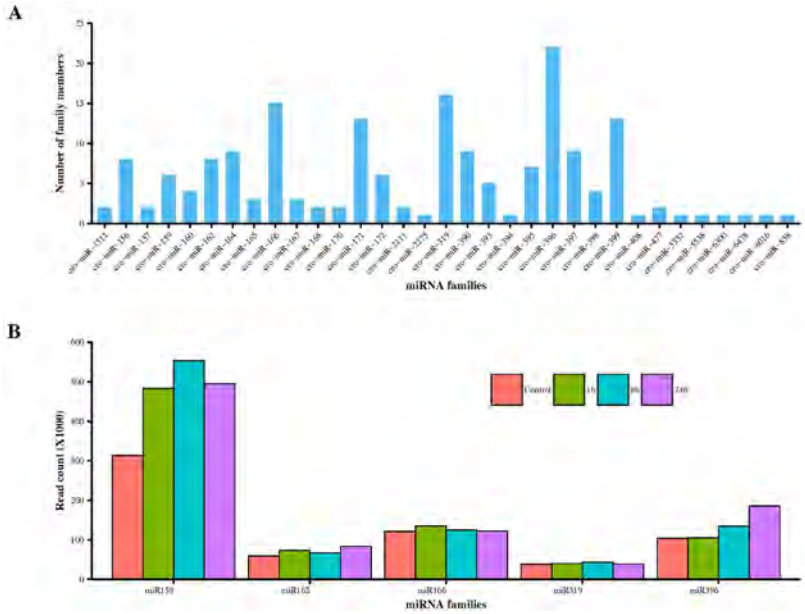

Figure 2. Number of members and abundance of known miRNA families identified in *C. roseus*. (**A**) Number of members in each conserved miRNA family in *C. roseus*. (**B**) Sequence read counts of top five conserved miRNA families in the small RNA libraries. The transcript abundance of each family in the control, as well as 1 h-, 8 h-, and 24 h-MeJA treated library is shown.

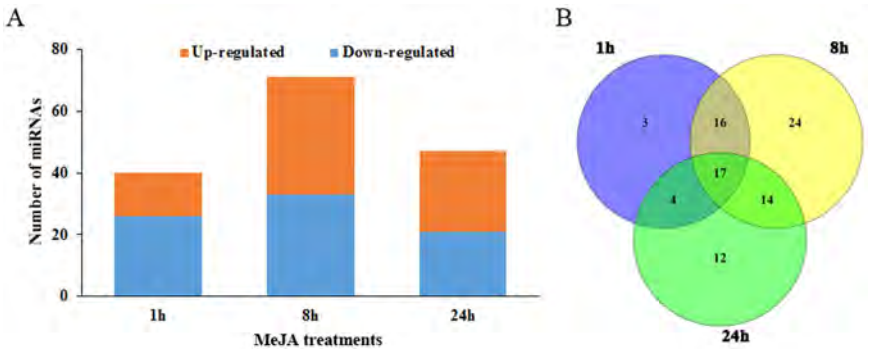

Figure 3. Differential expression analysis of miRNAs in *C. roseus* seedlings. (A) Numbers of up- and downregulated miRNAs in *C. roseus* seedlings after MeJA-treatment for 1 h, 8 h, or 24 h (**B**) Venn diagram showing the overlap of differentially expressed miRNAs (DEMs) between the three time points.

of 11 miRNAs, cro-miR319a-5p, cro-miR164b, cro-miR390b-3p-1, cro-miR164a, cro-miR393d, cro-novel-62, cro-novel-92, cro-novel-73, cro-novel-65, cro-novel-10, and cro-novel-63, were increased after MeJA treatment in all time-points. In contrast, 6 miRNAs, cro-novel-64, cro-novel-54, cro-novel-130, cro-miR398f, cro-miR319a-3p.2–3p, and cro-miR319a, were downregulated at all time-points.

Venn-diagram analysis revealed that 17 DEMs were common in all three time-points, whereas 16, 14, and 4 DEMS were common in 1 h–8 h, 8 h–24 h, and 1 h–24 h comparisons, respectively (Fig. 3B and Supplementary Table S9). Among the 17 common DEMs, 7 miRNAs (cro-novel-73, cro-novel-92, cro-novel-62, cro-novel-10, cro-miR393d, cro-miR164a, and cro-miR319a-5p) were upregulated and 5 miRNAs (cro-miR319a-3p.2-3p, cro-miR319a, cro-miR398f, cro-novel-64, and cro-novel-130) were downregulated at all three time points (Fig. 3B). In addition, the expression of five miRNAs (cro-novel-71, cro-novel-38, cro-novel-98, cro-miR168a, and cro-miR160h) were down 1 h following MeJA treatment, but were up after 8 h and 24 h. The DEMs were divided into four groups according to their expression patterns (Fig. 4A). The mean expression of DEMs in group-I (n = 30) increased after MeJA-treatment, peaking around 8 h. Those in group-II (n = 21) were initially upregulated by MeJA but gradually downregulated from 8 h to 24 h. The members of group-III (n = 17) were downregulated in response to MeJA; however, some members, including cro-novel-2, cro-miR396, and cro-miR396a-3p-4, were upregulated significantly between 8 h and 24 h. The mean expression of DEMs in group-IV (n = 21) increased continuously for 24 h (Fig. 4B).

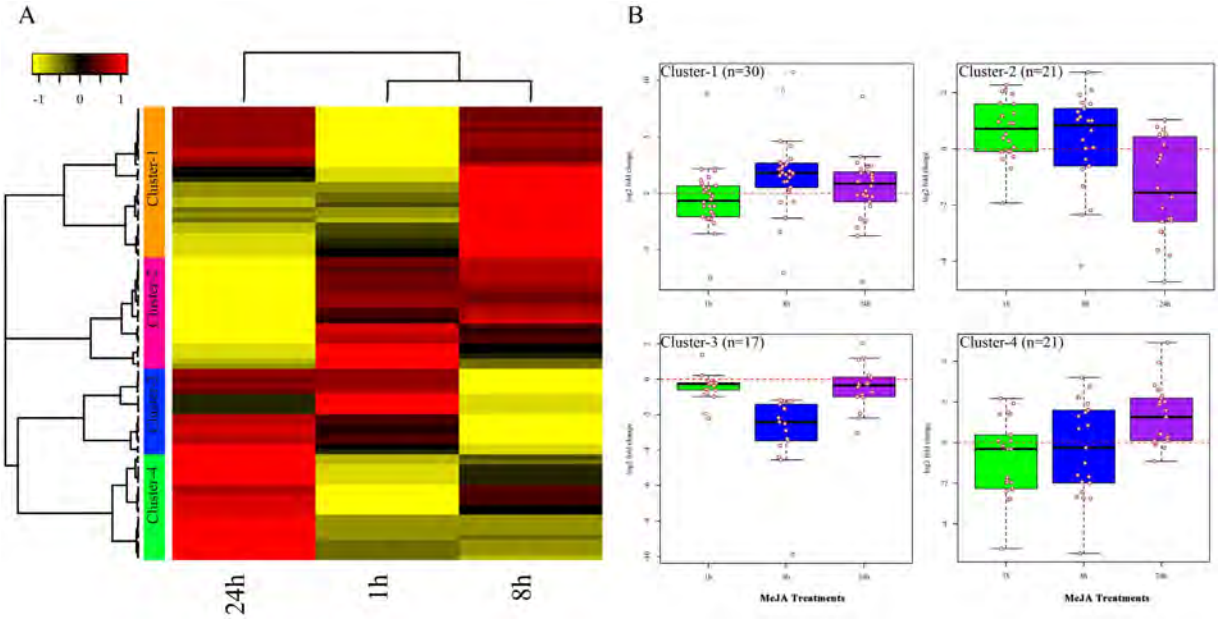

Figure 4. Hierarchical cluster analysis of DEMs in *C. roseus* seedlings. (**A**) Heat-map of the log2-fold change values of DEMs after 1 h, 8 h, and 24 h of MeJA treatment compared to the control. DEMs were divided into four different clusters (indicated by color-coded bars with numbers) based on their expression patterns. Columns denote the time points (1 h, 8 h, and 24 h) of MeJA treatment. The scale of heat-map is given as log2 fold change with a range from −1.0 (yellow) to +1.0 (red). (**B**) Box plots represent the log2 fold change (MeJA-treatment/control) in gene expression after MeJA treatment in four different clusters. Each single dot represents a miRNA.

Identification and classification of cro-miRNA targets. In order to explore the roles of DEMs in diverse biological processes, their putative targets were predicted using the open source web server, psRNATarget[48], with default parameters. The transcript sequences of the *C. roseus* genome were used as a reference set. Detailed information related to the predicted targets for conserved and *C. roseus*-specific miRNAs were included in Supplementary Table S10. A total of 519 candidate target genes were identified for 80 cro-miRNAs (149 target genes for 33 conserved miRNA and 374 target genes for 47 novel miRNAs, respectively), with an average of 6 targets per miRNA. The majority of the target genes (65.3%) were predicted to be regulated by transcript degradation, whereas the remaining targets are regulated by translational repression. Furthermore, 55 genes were targeted by more than one cro-miRNA. Among the conserved miRNA targets, many encode TFs, such as MYB TFs (targeted by cro-miR159), NAC domain-containing proteins (targeted by cro-miR164), and auxin response factors (regulated by cro-miR160). In addition, genes encoding receptor-like protein kinase, pentatricopeptide repeat (PPR) and tetratricopeptide repeat (TPR)-like superfamily proteins, and major facilitator superfamily proteins, were also identified as potential targets of cro-miR396 and cro-miR398. Potential targets were also identified for 47 *C. roseus*-specific miRNAs. These targets included mRNA encoding receptor-like protein kinase, ubiquitin protein ligase 6, blue-copper-binding protein, sterol methyltransferase 2, and F-box family protein. In addition, some *C. roseus*-specific miRNAs were also found to target TFs, including basic bHLH, bZIP domain proteins, and ARF family TFs (Supplementary Table S10).

The potential targets of the DEMs were annotated to better understand the biological functions of cro-miRNAs. Gene ontology (GO) analysis revealed that these target genes could be classified into 11 biological processes, 8 molecular functions, and 5 cellular components (Fig. 5). For biological processes, "cellular process", "multicellular organism development", and "response to stress", were the three most dominant GO categories. With regard to cellular components, "intracellular' and 'membrane" were the two most abundant GO terms. The GO term "intracellular" was significantly enriched for two children GO terms, "nucleus" and "cytoplasm". The three most dominant GO terms in molecular functions were "binding", "catalytic activity", and "transferase activity". Analysis of the GO term 'transcription factor activity' revealed that several families of TFs, such as GRAS, MYB, ARF, NAC, and GRF, were more dominant in this category. A significant number of genes associated with 'kinase activity' (mostly receptor-like kinases) were also found to be targeted by the cro-miRNAs. The GO term "catalytic activity" was enriched with children GO terms, "transferase activity" and "hydrolase activity".

Previous studies suggest that auxin negatively affects the expression of key TIA pathway genes in *Catharanthus*[18,19]. However, the underlying molecular mechanism of IAA-mediated repression is not well studied. ARFs are key components in auxin signaling pathway. Our target prediction analyses revealed *CrARF10*, *CrARF16,* and *CrARF17* as potential targets of MeJA-induced miR160. *HAM3*, a GRAS domain TF, was also found to have a high target prediction score in our analysis. We performed PPM-RACE on three selected targets, *ARF10*, *ARF16*, and HAM3 to demonstrate that they are indeed targeted by the respective miRNAs. Cloning of the PCR products, followed by sequencing of the PPM-RACE products, confirmed that *ARF10* and *ARF16* were

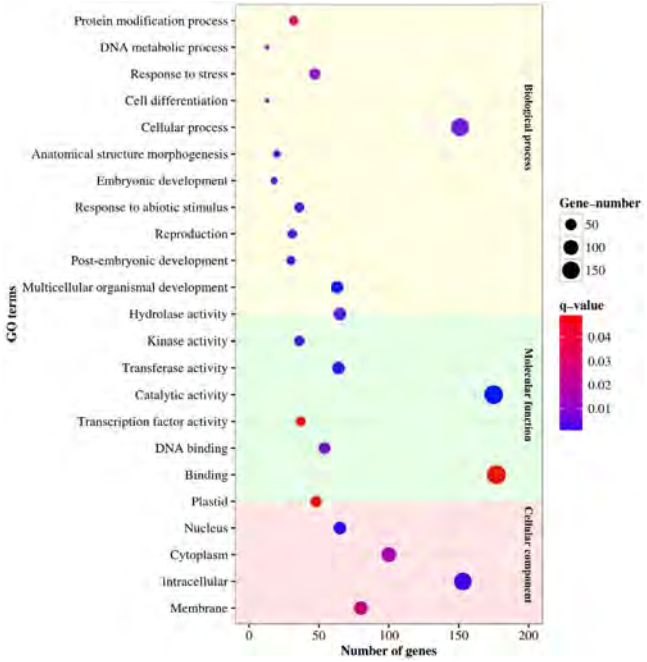

Figure 5. Gene ontology (GO) analysis of target genes of DEMs in *C. roseus* seedlings. Each GO term is represented by a single circle, that the color indicates the q-values and the significance of the GO term, and the size is proportional to the gene numbers. The Y-axis represents the names of enrichment GO terms. The X-axis represents the number of genes.

cleaved by the conserved cro-miR160, whereas the GRAS domain TF HAM3 was cleaved by cro-miR171b-3p-2, in *C. roseus* cells (Fig. 6A).

Expression profiles of DEMs and their targets. We used qRT-PCR to validate the small RNA sequencing (sRNA-seq) results of the DEMs and their potential targets. Expression of eleven (5 conserved and 6 novel) DEMs (Fig. 6B) and thirteen corresponding targets were measured using qRT-PCR (Fig. 6C). Expression of *cro-miR393d*, *cro-miR164a*, and *cro-miR164b* were upregulated, and that of cro-miR168a was downregulated, following 1 h MeJA treatment. There was no significant change in the expression of *miR160a* in response to 1 h MeJA treatment. The expression of all the selected conserved miRNAs were induced following 8 h and 24 h MeJA treatments. All analyzed *C. roseus*-specific miRNA were upregulated after 8 h and 24 h JA treatments. These results are in good agreement with the sRNA-seq data.

Usually, miRNAs and their target genes are expected to have contrasting expression patterns. We thus analyzed the expression of a number of TF genes (*ARF10, ARF16*, *ARF17*, *TIR1, AFB3*, *NAC1*, *NAC5* and *GRAS2*) which were predicted to be targeted by conserved and novel miRNAs, including cro-miR160, cro-miR3953d, cro-miR164, cro-novel-43, and cro-novel-38. As expected, the expression of the conserved and novel cro-miRNAs were inversely correlated to those of their targets (Fig. 6C).

Negative regulation of TIA structural and regulatory genes by auxin. TIA biosynthesis is influenced by a number of phytohormones including auxin and MeJA. Our results showed that expression of *CrARF10*, *CrARF16*, and *CrARF17* were repressed in MeJA-treated *C. roseus* seedlings. In addition, these three ARFs were identified as targets of MeJA-induced miRNA cro-miR160. The expression of *TIR1* and *AFB3*, crucial components in auxin signaling, were also altered in the MeJA-treated seedlings.

Auxin has been shown to represses the expression of several TIA pathway genes, including *TDC* and *STR*, in *C. roseus* cell lines[18,19]. Previous findings, and our analysis of JA-treated *C. roseus* seedlings, confirmed that most TIA biosynthetic pathway genes are induced by exogenous application of JA (Supplementary Fig. S4). We thus hypothesized that JA and auxin act antagonistically to regulate the TIA pathway in *C. roseus.* To test our hypothesis, we analyzed the expression of key regulatory and structural genes in *C. roseus* seedlings treated with IAA for different time periods. As shown in Fig. 7, auxin repressed the expression of key regulatory (*ORCA3*, *ORCA4*, *ORCA5*, *BIS1*) and structural genes (*STR*, *TDC*, *G10H*, *SLS*) in the TIA pathway. The transcript levels of *BIS1, ORCA4, SLS,* and *G10H* were significantly decreased at all time-points following IAA treatment. The expression of *ORCA3* was not affected by IAA at 1 h, but reduced after a longer treatment. The expression of *TDC*, *SGD,* and *STR,* were moderately affected by IAA treatment (Supplementary Fig. S5). In addition, we also measured the expression of two ARFs, *CrARF10* and *CrARF16,* in IAA-treated *Catharanthus* seedlings using qPCR. Expression of *CrARF10* and *CrARF16* were induced significantly following IAA treatment (Supplementary Fig. 6).

The repression of TIA pathway genes by IAA led us to hypothesize that ARFs repress TIA pathway genes by binding to key pathway gene promoters. To test our hypothesis, the promoter sequences of *TDC*, *G10H,* and *STR*

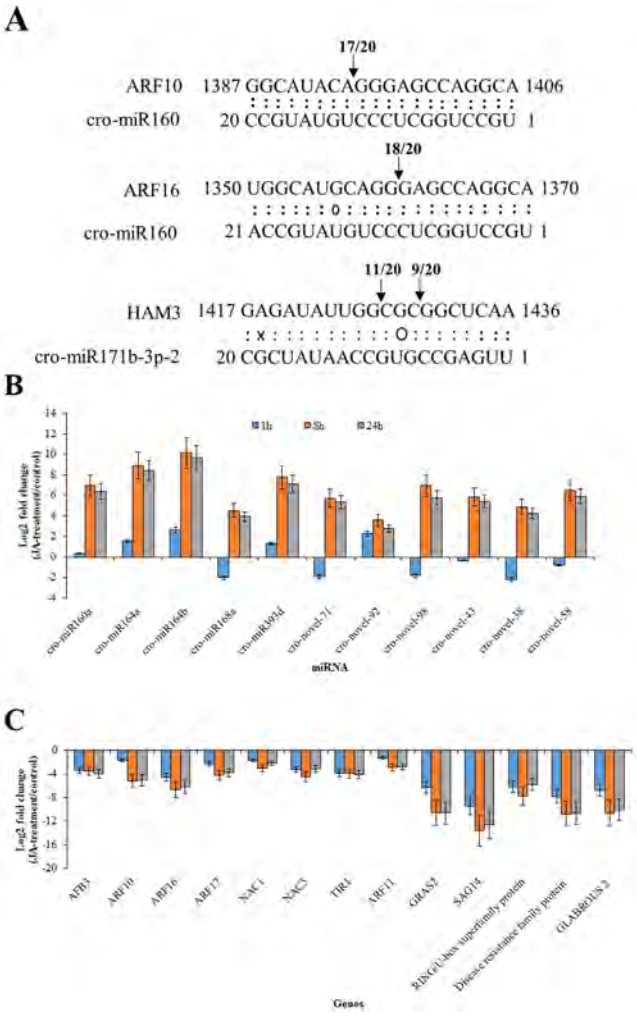

Figure 6. PPM-RACE and experimental validation of selected conserved and novel cro-miRNAs, as well as their predicted targets. (A) PPM-RACE confirmed the cleavage of *CrARF10* (CRO_T007962) and *CrARF16* (CRO_T023928) by cro-miR160 and that of HAM3 (CRO_T018940) by cro-miR171b-3p-2. cro-miR171b-3p-2 cleaves *HAM3* at two sites. The numbers above the sequences indicate the numbers of cleaved fragments detected at the cro-miR160 or cro-novel-43 target sites relative to the total fragments sequenced. For example, of 20 sequenced PCR fragments of ARF10, 17 were cleaved at position 1394 by cro-miR160. Watson-Crick pairing (:), G–U wobbles (O), and mismatched base pairing (X) are indicated. **(B,C)** Quantitative RT-PCR validated the expression of selected conserved and *C. roseus*-specific miRNAs (**B**) and their targets (**C**). The expression values of miRNAs and their targets were normalized against the expression of the endogenous controls, U6 snRNA and RPS9, respectively. The log2-transformed fold changes in mRNA levels (MeJA-treated/control) of miRNAs and their targets is shown. The data represent the mean values ± SD of three biological replicates.

were PCR-amplified, from *Catharanthus* genomic DNA using gene-specific primers, and cloned into a plant expression vector containing a firefly luciferase reporter[9]; the promoter-driven transcriptional activities were measured, alone or in the presence of CrARF16, in a tobacco protoplast assay. As shown in Fig. 8, all three promoters individually generated significant background luciferase activity. However, co-electroporation of the promoters-reporter plasmids with CrARF16 significantly repressed the basal activities of all three promoters.

Discussion

Here, we report the identification, expression analysis, target prediction and validation of conserved and novel miRNAs in *C. roseus* seedlings. Additionally, we identified a set of JA-responsive cro-miRNAs and attempted to explore their potential roles in posttranscriptional control of TIA biosynthesis.

The miRNAome of *C. roseus*. Using high-throughput sequencing, we identified 354 cro-miRNAs (Supplementary Tables S2, S3, S6 and S7), a number that is close to what have been reported from *Arabidopsis* (427), *Zea mays* (321), *Sorghum bicolor* (241), and *Populus trichocarpa* (401), but less than those from *Medicago truncatula* (756), *Oryza sativa* (713), and *Glycine max* (639). A previous study has identified 81 conserved miRNAs in *Catharanthus* seedlings[40]. Most of the conserved miRNA family members identified in that study are present

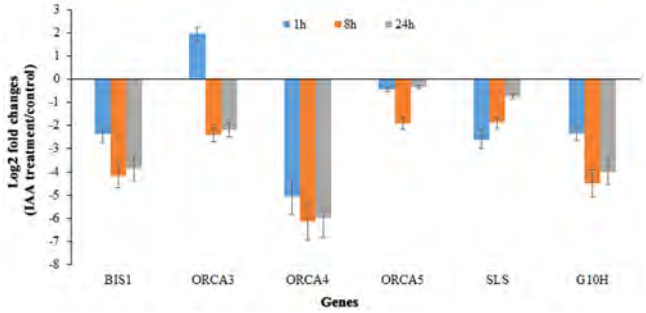

Figure 7. Quantitative RT-PCR analysis of selected TIA biosynthetic pathway genes in control and IAA-treated *C. roseus* seedlings. The relative abundance of individual gene is presented as the ratio of the IAA-treated vs. control seedlings. The expression value of each gene was normalized against the expression of endogenous control, RPS9. The log2-transformed fold changes in mRNA levels (IAA-treated/control) of the target genes is shown. The data represent the mean values ± SD of two biological replicates.

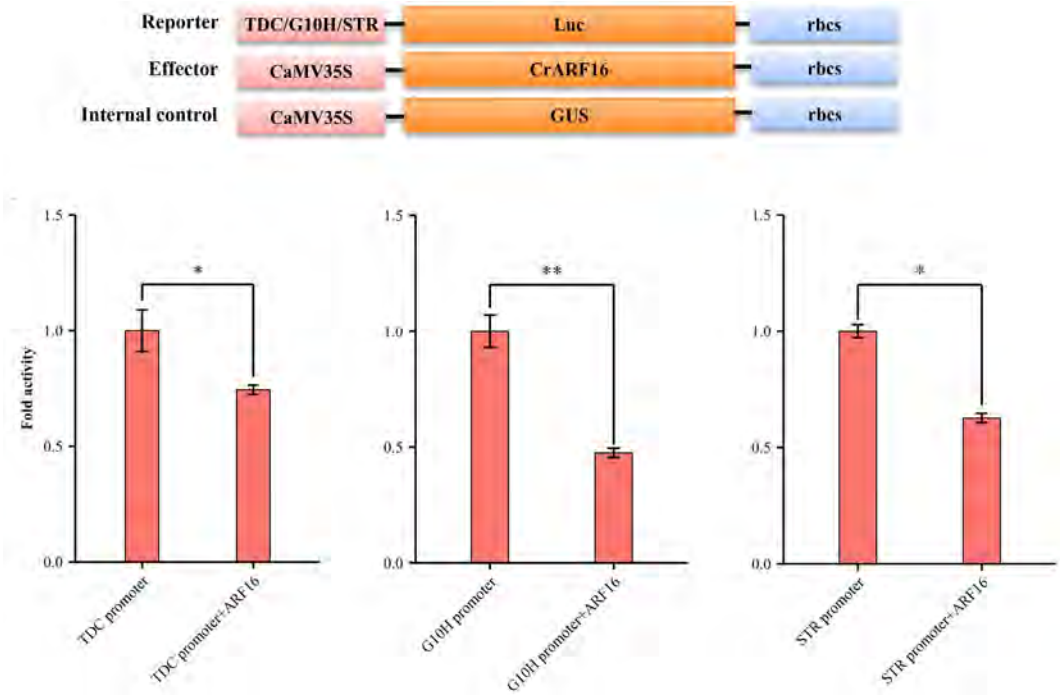

Figure 8. Repression of *TDC*, *G10H*, and *STR* promoter activities in tobacco cells by CrARF16. *TDC*, *G10H*, and *STR* promoters fused to the *fire-fly* luciferase reporter were electroporated into tobacco protoplasts either alone or with the effector plasmid expressing *CrARF16*. A plasmid containing the *ß-glucuronidase* (*GUS*) reporter, controlled by the *CaMV*35S promoter and *rbcS* terminator, was used as a normalization control. Schematic diagrams of the reporter, effector, and internal control plasmids used in this assay are shown on the top. Luciferase and GUS activities were measured 20 h after electroporation. Luciferase activity was normalized against the GUS activity. The reporter alone without effectors served as the control. Data presented are means ± SDs of three biological replicates. Asterisks indicate a significant difference compared to the control at $^{*}P < 0.08$ or $^{**}P < 0.005$ (t-test).

in our dataset. The 24 nt long sRNAs dominated the *C. roseus* sRNA transcriptome, indicating the dominance of heterochromatic small interfering RNA (siRNA) (Fig. 1A,B). Similar observations have been made for many other plant species, including peanut, hot pepper, cucumber, rice and trifoliate orange[49–53]. The high percentage of 24 nt sRNAs probably plays a vital role in preserving genome integrity by heterochromatic-histone modification[54]. Our dataset also shows a high ratio of total reads/unique reads for the 21 nt class. The majority of the identified cro-miRNAs were 21 nt in length, which is the canonical size for miRNAs generated from DCL1 processing[55]. Based on the recently published draft *C. roseus* genome, which is not fully assembled and annotated, we were able to predict 65 precursor sequences representing 28 conserved miRNAs in *C. roseus* genome (Supplementary Table S3). Cro-miR159, -miR396, -miR166, -miR162, and -miR319 are highly abundant in *C. roseus* (Supplementary Table S4). MiR159 and miR319 are conserved family of miRNA and important for plant

growth, morphogenesis, and reproduction, by regulating the expression of a number of MYB and TCP family TF genes[56]. The conserved miR396 regulates the GROWTH-REGULATING FACTOR (GRF) family TFs, which control cell proliferation in *Arabidopsis* leaves[57]. MiR162 targets DICER-LIKE1 (DCL1), which catalyzes the formation of miRNA sequences, thus controlling miRNA biosynthesis by negative feedback regulation[58]. MiR166 and miR159 are involved in abiotic stress responses such as cold and salinity[59,60]. A previous study has identified conserved miRNAs from 99 different tissues in 34 different plant species[61]. We show that the *C. roseus* miRNAome harbors most of the ancient miRNA families, including miR160, miR159, and miR164 (Fig. 2A, Supplementary Table S2). In addition to the conserved miRNAs, all sequenced and analyzed plant genomes contain family- or species-specific miRNAs that may have originated and diverged on scales ranging from family to species[62]. The *C. roseus* genome harbors numerous miRNAs that lack close orthologs in other plant species (Supplementary Table S7). Consistent with previous observations[63,64], we also found that the majority of the *C. roseus*-specific miRNAs show relatively low expression when compared to the conserved miRNAs (Supplementary Table S8). A previous study[40] has identified a limited number of cro-miRNAs that is significantly lower than the average numbers of miRNAs reported in other plant species. The miRNAome reported here provides a comprehensive account of the numbers and expression profiles of conserved and novel miRNAs in *C. roseus* seedlings. Unlike the previous report, the majority of the cro-miRNAs identified in the study are mapped to the *C. roseus* draft genome. Moreover, expression analysis of the selected conserved and novel cro-miRNAs by qRT-PCR complements the sRNA-seq data and validates our miRNA prediction criteria (Fig. 6A–C).

JA-responsive miRNAs target key regulators in auxin signaling. JA-responsive expression of regulatory and structural genes is a hallmark of TIA pathway. Through modulating the expression of the TIA pathway genes, exogenous application of JA increases the production of TIAs in *C. roseus* seedlings, hairy roots, and cell cultures[65–67]. A number of JA-responsive regulatory genes have been isolated and characterized for their roles in regulation of the TIA pathway. However, the influence of JA on miRNA expression is not well studied. In *Taxus chinensis,* MeJA downregulates *miR156*, *miR168*, *miR169*, *miR172*, *miR172*, *miR396*, *miR480*, and *miR1310,* but upregulates *miR164* and *miR390*[68]. Our analysis shows that a number of cro-miRNAs were also differentially expressed in response to MeJA treatment (Fig. 3A,B; Supplementary Table S9) and the DEMs were divided into four independent clusters based on their expression (Fig. 4A,B). We observed a significant induction of *cro-miR160*, *cro-miR168*, and *cro-miR393* after JA treatment. Target prediction of MeJA-responsive DEMs identified 519 potential targets (Supplementary Table S10). GO analysis led to a better understanding of their biological functions (Fig. 5). Detailed analysis of the GO term, "transcription factor activity" revealed the significance of several auxin signaling genes, including *TIR1, CrARF10*, *CrARF16*, and *CrARF17*.

ARFs are a plant-specific TF family that controls auxin-regulated transcription. Most ARFs have a conserved N-terminal DNA binding domain, C-terminal dimerization domain, and a non-conserved middle region that confers transcriptional repression or activation[69,70]. ARFs bind to AuxREs, which are found in the promoters of early auxin response genes, including *Aux/IAA*, *SMALL AUXIN UPREGULATED RNA* (*SAUR*), and *GH3*, to either enhance or repress their transcription[71,72]. In *Arabidopsis, ARF10*, *ARF16*, and *ARF17* are reported to be targeted by miR160[73]. We observed a significant upregulation in *cro-miR160* after MeJA treatment (Fig. 6B). As expected, expression of *CrARF10*, *CrARF16*, and *CrARF17* were significantly downregulated after MeJA-treatment in *C. roseus* seedlings (Fig. 6C). Other than *cro-miR160*, MeJA also induced the expression of *cro-miR164a*, *cro-miR164b*, *cro-miR393d,* and *cro-novel-92,* which are predicted to target the auxin signaling genes, including *NAC1, NAC5, TIR1, AFB3,* and *ARF11.* The JA-responsive differential expression and targeting of IAA signaling genes by these MeJA-induced cro-miRNAs suggest that they can potentially affect TIA biosynthesis through regulating key factors in IAA signaling.

Cro-miRNA-targeted ARFs are potentially involved in regulation of the TIA pathway. Previous studies have reported the negative effects of auxin on expression of several structural genes in the TIA pathway[18,19]. However, the molecular mechanism underlying auxin-mediated repression of TIA pathway genes is still elusive. Our results not only confirmed the previous findings, but also showed that auxin significantly downregulates the expression of several key transcriptional regulators, including *ORCA3*, *ORCA4*, and *BIS1*, in TIA biosynthetic pathway (Fig. 7). In addition, we showed that auxin upregulates the expression of two ARFs, *CrARF10* and *CrARF16*, in *Catharanthus* seedlings. We also demonstrated that the miRNA-targeted CrARF16 represses the activity of key TIA pathway gene promoters, such as *TDC*, *STR,* and *G10H* in plant cells (Fig. 8). Taken together our findings suggest that auxin and JA act antagonistically to regulate the expression of TIA pathway genes, in part, through the posttranscriptional regulation by cro-miRNAs. Auxin represses the expression of key TIA pathway genes, possibly mediated by the CrARF repressors. MeJA-induced expression of cro-miRNAs result in the degradation of CrARF repressors, leading to activation of TIA pathway genes.

In conclusion, this study provides a comprehensive account of the *C. roseus* miRNAome and the possible roles of cro-miRNAs in regulating the biosynthesis of TIAs. In addition, targeting of CrARFs by MeJA-induced cro-miRNAs to attenuate the repression of auxin highlights antagonistic functions of two key phytohormones in TIA pathway regulation. Our findings provide a starting point for further investigation of the regulatory roles miRNAs in specialized metabolite biosynthesis, in particular, TIAs in *C. roseus*.

Materials and Methods

Plant Materials, treatments, and RNA isolation. *Catharanthus roseus* (L.) G. Don cv. 'Cooler Apricot' seeds were surface-sterilized and germinated on half-strength Murashige and Skoog (MS) basal medium. Two week-old seedlings were treated with 100 μM MeJA or 10 μM indole acetic acid (IAA) for 1 h, 8 h, or 24 h and frozen immediately in liquid nitrogen. Mock-treated seedlings were used as controls. Total RNA were isolated from 100 mg of control and MeJA-treated seedlings using miRVana miRNA isolation Kit with phenol (ThermoFisher

Scientific, USA). RNA quantity was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Quality of the RNA samples was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RNA integrity number (RIN) above 8 were used for library preparation. For expression analysis of regulatory and structural genes in the TIA pathway, RNA were isolated from the control and MeJA- or IAA-treated seedlings using RNeasy plant mini kit (QIAGEN, USA).

High-throughput sequencing of small RNAs. Two micrograms of RNA from each sample was sent to the Sequencing and Genotyping Center at the Delaware Biotechnology Institute at the University of Delaware for small RNA library preparation and sequencing. The libraries were pooled together and sequenced on an Illumina HiSeq 2500. Deep sequencing was performed in triplicates for each treatment for a 50 cycle single end run. The data quality was checked at the Sequencing and Genotyping Center and sequencing reads were provided in the FASTq format.

Analysis of small RNA sequencing data. Low quality and contaminated reads were removed as described previously[74]. Sequences smaller than 18 nucleotide (nt) and larger than 24 nt were also removed. Reads ranging from 18 to 24 nt in length, were mapped to the *C. roseus* reference genome sequence[41] with the software, Bowtie2[75], with no mismatches allowed. The unmapped sequences were removed from analysis. Reads, matched with the *C. roseus* genome, were compared with non-coding sRNAs deposited in NCBI GenBank and RNA family (Rfam) databases. Reads that matched ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nucleolar RNAs (snoRNAs), and small nuclear RNAs (snRNAs), were filtered and removed. The remaining reads were used for downstream analyses.

Identification of conserved and *C. roseus*-specific (novel) miRNAs. For conserved cro-miRNA identification, sequence reads were aligned with known miRNA sequences from other plant species deposited in miRBase 21 (http://www.mirbase.org/index.shtml)[42,76] with a maximum of two mismatches. The unaligned reads were then subjected to miR-PREFeR pipeline to predict novel miRNA candidates. miR-PREFeR[45] utilizes expression patterns of miRNAs and follows the criteria for plant miRNA annotation[46] to accurately predict plant miRNAs from one or more small RNA-seq samples. The pipeline generates candidate regions and candidate mature sequences of each candidate region based on alignment depth of 20 reads. Predicted novel cro-miRNAs were further screened and validated in this study. The secondary structures of precursors for all conserved and novel cro-miRNA candidates were constructed using the RNAfold software[77].

Differential expression analysis of JA responsive cro-miRNAs. Differential expression of cro-miRNAs was calculated from read count data using DESseq2 of the R package as described[78]. Cro-miRNAs with log2 fold change differences ≥ 1, p-value ≤ 0.05, and false discovery rate (FDR) ≤ 0.1, were considered to be differentially expressed. The R package 'gplots' was used for heat map generation of differentially expressed cro-miRNAs. Normalized count was calculated as described previously[44].

Prediction and annotation of cro-miRNA target genes. The potential target prediction of all identified cro-miRNAs was conducted using the plant small RNA target server (psRNATarget; http://plantgrn.noble.org/psRNATarget/), a widely used web-based tool[48]. Candidate targets were analyzed by locally installed BLASTX search against the NCBI Nr database with the default parameters. BiNGO 3.0.3 plug-in[79] of Cytoscape[80] was used for gene ontology (GO) analysis of target genes of JA responsive cro-miRNAs.

Quantitative RT-PCR validation of selected, differentially expressed cro-miRNAs and their potential targets. Expression of cro-miRNAs were analyzed using the poly(T) adaptor RT-PCR method[81]. One μg of RNase-free DNase I treated total RNA was polyadenylated at 37 °C for 60 min in a 10 μl reaction volume containing 0.08 units poly (A) polymerase. The polyadenylated RNA was then reverse transcribed in a 20 μl reaction mix with SuperScript III Reverse Transcriptase (Invitrogen, USA) and oligo (dT) adaptor, following the manufacturer's instructions. For expression analysis of regulatory and structural genes in TIA pathway, synthesis of first strand cDNA from total RNA, and Quantitative RT-PCR (qRT-PCR), were performed as described previously[13]. The comparative cycle threshold (Ct) method (bulletin no. 2; Applied Biosystems, http://www.appliedbiosystems.com) was used to measure the transcript levels. U6 small nuclear ribonucleic acid (snRNA) and Ribosomal Protein S9 (RPS9), were used as the normalization controls for miRNA and target mRNA, respectively. The primers used in RT-qPCR are listed in Supplementary Table S11. All experiments were performed using two biological replicates with three technical replicates. Specificity of miRNA primers was determined by cloning the PCR products into the pGEM-T Easy vector (Promega, USA), followed by sequencing.

Poly(A) polymerase-mediated rapid amplification of cDNA ends. Poly(A) polymerase-Mediated Rapid Amplification of cDNA Ends (PPM-RACE) was conducted using the protocol described previously[82] to map the cleavage sites of target transcripts. Briefly, total RNA (2 μg) isolated from *C. roseus* seedlings were polyadenylated with poly (A) polymerase and used for cDNA synthesis using oligodT primer with an adapter. The first strand cDNA served as template to amplify the cleaved products using adapter and gene-specific primers. The PCR products were cloned into the pGEM-easy vector and sequenced.

Transcriptomic Analysis of MeJA-treated *C. roseus* tissues. To analyze the effect of MeJA on *C. roseus* transcriptome, publically available RNAseq data were obtained from the sequence read archive database (accession number PRJNA185483). Raw reads were cleaned and filtered as described previously[74]. Finally, cleaned reads were mapped to the *C. roseus* reference sequence[41] with Bowtie2[75], and Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values were calculated by eXpress[83].

Plasmid construction, protoplast isolation and electroporation. For the protoplast assay, reporter plasmids were generated by cloning the *TDC, STR,* or *G10H* promoters into a pUC vector containing the fire-fly *luciferase* and *rbcS* terminator. The effector plasmids were generated by cloning *CrARF16* into a modified pBlue-Script vector containing the cauliflower mosaic virus (CaMV) 35S promoter and *rbcS* terminator. A plasmid containing ß-glucuronidase (*GUS*) reporter, controlled by the *CaMV*35S promoter and *rbcS* terminator, was used as an internal control in the protoplast assay. The reporter plasmids were electroporated, alone or with the effector plasmid, into tobacco protoplasts. Protoplast isolation from tobacco cell suspension cultures, electroporation, and measurement of luciferase and GUS activities in protoplasts were performed as descried previously[84].

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Author Contributions

L.Y. and S.P. designed research; E.S., S.S., B.P., J.G. and P.P. performed experiments; E.S., S.S. and S.P. analyzed data; S.S., S.P. and L.Y. wrote the paper.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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FISCAL YEAR 2017 – 2018

FINANCIAL REPORT

JULY 1, 2017 – SEPTEMBER 30, 2017

QUARTERLY REPORT

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TOBACCO RESEARCH INCOME
INCOME COMPARISON

Fiscal Years	2011-2012	2012-2013	2013-2014	2014-2015	2015-2016	2016-2017	2017-2018
July	$ 12,468.00	$ 23,478.65	$ (29,962.52)	$ 13,386.52	$ 139,619.47	$ 157,187.49	$ 2,459.48
August	$ 13,255.13	$ 185,546.95	$ 343,968.71	$ 301,292.71	$ 177,916.68	$ 137,652.03	$ 292,266.42
September	$ 483,727.76	$ 142,871.25	$ 152,700.22	$ 133,527.76	$ 47,768.58	$ 42,873.59	$ 139,414.92
1st QUARTER	**$ 509,450.89**	**$ 351,896.85**	**$ 466,706.41**	**$ 448,206.99**	**$ 365,304.73**	**$ 337,713.11**	**$ 434,140.82**
October	$ 129,904.00	$ 176,915.29	$ 148,858.82	$ 166,587.97	$ 255,006.22	$ 157,120.53	
November	$ 90,126.34	$ 231,929.57	$ 55,176.52	$ 74,462.42	$ 127,495.52	$ 251,055.77	
December	$ 141,353.49	$ 218,829.28	$ 136,860.86	$ 190,289.54	$ 26,196.02	$ 113,251.82	
2nd QUARTER	**$ 361,383.83**	**$ 627,674.14**	**$ 340,896.20**	**$ 431,339.93**	**$ 408,697.76**	**$ 521,428.12**	**$ -**
SIX MONTHS	**$ 870,834.72**	**$ 979,570.99**	**$ 807,602.61**	**$ 879,546.92**	**$ 774,002.49**	**$ 859,141.23**	**$ 434,140.82**
January	$ 148,008.05	$ 143,268.63	$ 258,621.49	$ 44,597.62	$ 264,622.53	$ 109,584.57	
February	$ 134,668.72	$ 11,634.70	$ 115,623.41	$ 212,408.73	$ 10,472.72	$ 155,644.33	
March	$ 219,397.93	$ 194,538.93	$ 57,626.04	$ 133,593.90	$ 255,769.54	$ 159,012.56	
3rd QUARTER	**$ 502,074.70**	**$ 349,442.26**	**$ 431,870.94**	**$ 390,600.25**	**$ 530,864.79**	**$ 424,241.46**	**$ -**
NINE MONTHS	**$ 1,372,909.42**	**$ 1,329,013.25**	**$ 1,239,473.55**	**$ 1,270,147.17**	**$ 1,304,867.28**	**$ 1,283,382.69**	**$ 434,140.82**
April	$ 120,211.36	$ 240,079.82	$ 243,424.06	$ 165,299.42	$ 20,461.50	$ 43,764.59	
May	$ 180,321.06	$ 81,297.53	$ 24,607.31	$ 183,052.97	$ 144,713.80	$ 174,933.87	
June	$ 325,362.14	$ 282,726.79	$ 296,886.53	$ 137,563.73	$ 288,160.23	$ 242,003.23	
4th QUARTER	**$ 625,894.56**	**$ 604,104.14**	**$ 564,917.90**	**$ 485,916.12**	**$ 453,335.53**	**$ 460,701.69**	**$ -**
TOTAL INCOME	**$ 1,998,803.98**	**$ 1,933,117.39**	**$ 1,804,391.45**	**$ 1,756,063.29**	**$ 1,758,202.81**	**$ 1,744,084.38**	**$ 434,140.82**

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JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER

KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT

FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235410080	HOLDING ACCOUNT	All Budget Commitmen	-$ 2,000,000.00	-$ 294,725.90	-$ 139,414.92	-$ 434,140.82			$ -	-$ 1,565,859.18
1235410080	HOLDING ACCOUNT	All Revenue Commitme	-$ 2,000,000.00	-$ 294,725.90	-$ 139,414.92	-$ 434,140.82			$ -	-$ 1,565,859.18
1235410080	HOLDING ACCOUNT	TTL Revenue Excl Tra	-$ 2,000,000.00	-$ 294,725.90	-$ 139,414.92	-$ 434,140.82			$ -	-$ 1,565,859.18
1235410080	HOLDING ACCOUNT	State/Local Grants a	-$ 1,650,000.00	-$ 294,725.90	-$ 139,414.92	-$ 434,140.82			$ -	-$ 1,215,859.18
1235410080	HOLDING ACCOUNT	Fund Balance	-$ 350,000.00							-$ 350,000.00
1235410090	KENTUCKY TOBACCO RES	All Budget Commitmen	$ 1,000.00							$ 1,000.00
1235410090	KENTUCKY TOBACCO RES	All Expenses Commitm	$ 1,000.00							$ 1,000.00
1235410090	KENTUCKY TOBACCO RES	TTL Expense Excl Tra	$ 1,000.00							$ 1,000.00
1235410090	KENTUCKY TOBACCO RES	Operating Expense	$ 1,000.00							$ 1,000.00
1235410100	ADMINISTRATION	All Budget Commitmen	$ 251,522.00	$ 25,351.86	$ 16,192.21	$ 41,544.07	$ 81,325.71	$ 58.83	$ -	$ 128,593.39
1235410100	ADMINISTRATION	All Expenses Commitm	$ 251,522.00	$ 25,351.86	$ 16,192.21	$ 41,544.07	$ 81,325.71	$ 58.83	$ -	$ 128,593.39
1235410100	ADMINISTRATION	TTL Expense Excl Tra	$ 251,522.00	$ 25,351.86	$ 16,192.21	$ 41,544.07	$ 81,325.71	$ 58.83	$ -	$ 128,593.39
1235410100	ADMINISTRATION	Staff		$ 15,036.19	$ 10,288.28	$ 25,324.47	$ 61,776.90			-$ 87,101.37
1235410100	ADMINISTRATION	Other Personnel	$ 237,235.00	$ 4,355.40	$ 2,267.92	$ 6,623.32				$ 230,611.68
1235410100	ADMINISTRATION	Fringe Benefits		$ 4,530.49	$ 3,143.47	$ 7,673.96	$ 19,548.81			-$ 27,222.77
1235410100	ADMINISTRATION	Operating Expense	$ 14,287.00	$ 1,429.78	$ 492.54	$ 1,922.32	$ -	$ 58.83	$ -	$ 12,305.85
1235410110	KTRDC PERSONNEL	All Budget Commitmen	$ 928,630.00	$ 72,357.85	$ 67,854.92	$ 140,212.77	$ 354,293.37		-$ 44.72	$ 434,168.58
1235410110	KTRDC PERSONNEL	All Expenses Commitm	$ 928,630.00	$ 72,357.85	$ 67,854.92	$ 140,212.77	$ 354,293.37		-$ 44.72	$ 434,168.58
1235410110	KTRDC PERSONNEL	TTL Expense Excl Tra	$ 928,630.00	$ 72,357.85	$ 67,854.92	$ 140,212.77	$ 354,293.37		-$ 44.72	$ 434,168.58
1235410110	KTRDC PERSONNEL	Faculty		$ 14,681.54	$ 7,340.77	$ 22,022.31	$ 70,489.87			-$ 92,512.18
1235410110	KTRDC PERSONNEL	Staff		$ 39,769.20	$ 43,545.02	$ 83,314.22	$ 198,655.26			-$ 281,969.48
1235410110	KTRDC PERSONNEL	Other Personnel		$ 11.55		$ 11.55				-$ 11.55
1235410110	KTRDC PERSONNEL	Fringe Benefits		$ 16,635.56	$ 16,203.90	$ 32,839.46	$ 85,148.24			-$ 117,987.70
1235410110	KTRDC PERSONNEL	Operating Expense	$ 928,630.00	$ 1,260.00	$ 765.23	$ 2,025.23			-$ 44.72	$ 926,649.49

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER
KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT
FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235410120	PUBLICATIONS & TRAVE	All Budget Commitmen	$ 14,000.00	$ 1,947.11	$ 79.11	$ 2,026.22	$ 110.00		$ -	$ 11,863.78
1235410120	PUBLICATIONS & TRAVE	All Expenses Commitm	$ 14,000.00	$ 1,947.11	$ 79.11	$ 2,026.22	$ 110.00		$ -	$ 11,863.78
1235410120	PUBLICATIONS & TRAVE	TTL Expense Excl Tra	$ 14,000.00	$ 1,947.11	$ 79.11	$ 2,026.22	$ 110.00		$ -	$ 11,863.78
1235410120	PUBLICATIONS & TRAVE	Operating Expense	$ 14,000.00	$ 1,947.11	$ 79.11	$ 2,026.22	$ 110.00		$ -	$ 11,863.78
1235410130	BUILDING MAINTENANCE	All Budget Commitmen	$ 121,430.00	$ 927.25	$ 865.40	$ 1,792.65	$ -	$ -	$ -	$ 119,637.35
1235410130	BUILDING MAINTENANCE	All Expenses Commitm	$ 121,430.00	$ 927.25	$ 865.40	$ 1,792.65	$ -	$ -	$ -	$ 119,637.35
1235410130	BUILDING MAINTENANCE	TTL Expense Excl Tra	$ 121,430.00	$ 927.25	$ 865.40	$ 1,792.65	$ -	$ -	$ -	$ 119,637.35
1235410130	BUILDING MAINTENANCE	Operating Expense	$ 121,430.00	$ 927.25	$ 865.40	$ 1,792.65	$ -	$ -	$ -	$ 119,637.35
1235410140	KTRDC PRO CARD	All Budget Commitmen		$ 10,995.13	-$ 7,496.57	$ 3,498.56			-$ 279.87	-$ 3,218.69
1235410140	KTRDC PRO CARD	All Expenses Commitm		$ 10,995.13	-$ 7,496.57	$ 3,498.56			-$ 279.87	-$ 3,218.69
1235410140	KTRDC PRO CARD	TTL Expense Excl Tra		$ 10,995.13	-$ 7,496.57	$ 3,498.56			-$ 279.87	-$ 3,218.69
1235410140	KTRDC PRO CARD	Operating Expense		$ 10,995.13	-$ 7,496.57	$ 3,498.56			-$ 279.87	-$ 3,218.69
1235410180	SHOP	All Budget Commitmen	$ 1,000.00	$ 24.24	$ 255.52	$ 279.76			$ -	$ 720.24
1235410180	SHOP	All Expenses Commitm	$ 1,000.00	$ 24.24	$ 255.52	$ 279.76			$ -	$ 720.24
1235410180	SHOP	TTL Expense Excl Tra	$ 1,000.00	$ 24.24	$ 255.52	$ 279.76			$ -	$ 720.24
1235410180	SHOP	Operating Expense	$ 1,000.00	$ 24.24	$ 255.52	$ 279.76			$ -	$ 720.24
1235410240	LABORATORY EQUIPMENT	All Budget Commitmen	$ 20,000.00	$ 2,625.83	$ 3,916.37	$ 6,542.20	$ -	$ -		$ 13,457.80
1235410240	LABORATORY EQUIPMENT	All Expenses Commitm	$ 20,000.00	$ 2,625.83	$ 3,916.37	$ 6,542.20	$ -	$ -		$ 13,457.80
1235410240	LABORATORY EQUIPMENT	TTL Expense Excl Tra	$ 20,000.00	$ 2,625.83	$ 3,916.37	$ 6,542.20	$ -	$ -		$ 13,457.80
1235410240	LABORATORY EQUIPMENT	Operating Expense	$ 20,000.00	$ 2,625.83	$ 3,916.37	$ 6,542.20	$ -	$ -		$ 13,457.80
1235410250	UNALLOCATED RESERVE	All Budget Commitmen	$ 272,824.00							$ 272,824.00
1235410250	UNALLOCATED RESERVE	All Expenses Commitm	$ 272,824.00							$ 272,824.00
1235410250	UNALLOCATED RESERVE	TTL Expense Excl Tra	$ 272,824.00							$ 272,824.00
1235410250	UNALLOCATED RESERVE	Operating Expense	$ 272,824.00							$ 272,824.00

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER

KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT

FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235410280	GENERAL LABORATORY	All Budget Commitmen	$ 65,000.00	$ 1,102.98	$ 371.40	$ 1,474.38			$ -	$ 63,525.62
1235410280	GENERAL LABORATORY	All Expenses Commitm	$ 65,000.00	$ 1,102.98	$ 371.40	$ 1,474.38			$ -	$ 63,525.62
1235410280	GENERAL LABORATORY	TTL Expense Excl Tra	$ 65,000.00	$ 1,102.98	$ 371.40	$ 1,474.38			$ -	$ 63,525.62
1235410280	GENERAL LABORATORY	Operating Expense	$ 65,000.00	$ 1,102.98	$ 371.40	$ 1,474.38			$ -	$ 63,525.62
1235411040	DISCRETIONARY	All Budget Commitmen	$ 6,500.00	$ 260.84	$ 29.32	$ 290.16			$ -	$ 6,209.84
1235411040	DISCRETIONARY	All Expenses Commitm	$ 6,500.00	$ 260.84	$ 29.32	$ 290.16			$ -	$ 6,209.84
1235411040	DISCRETIONARY	TTL Expense Excl Tra	$ 6,500.00	$ 260.84	$ 29.32	$ 290.16			$ -	$ 6,209.84
1235411040	DISCRETIONARY	Operating Expense	$ 6,500.00	$ 260.84	$ 29.32	$ 290.16			$ -	$ 6,209.84
1235411310	OUTREACH & COMMUNICA	All Budget Commitmen	$ 20,000.00	$ 24,682.90	$ 2,692.92	$ 27,375.82	$ 1,162.00	$ -	$ 475.61	-$ 9,013.43
1235411310	OUTREACH & COMMUNICA	All Expenses Commitm	$ 20,000.00	$ 24,682.90	$ 2,692.92	$ 27,375.82	$ 1,162.00	$ -	$ 475.61	-$ 9,013.43
1235411310	OUTREACH & COMMUNICA	TTL Expense Excl Tra	$ 20,000.00	$ 24,682.90	$ 2,692.92	$ 27,375.82	$ 1,162.00	$ -	$ 475.61	-$ 9,013.43
1235411310	OUTREACH & COMMUNICA	Other Personnel		$ 4,680.46	$ 2,340.23	$ 7,020.69				-$ 7,020.69
1235411310	OUTREACH & COMMUNICA	Fringe Benefits		$ 395.40	$ 197.69	$ 593.09				-$ 593.09
1235411310	OUTREACH & COMMUNICA	Operating Expense	$ 20,000.00	$ 2,782.04	$ 155.00	$ 2,937.04	$ 1,162.00		$ 475.61	$ 15,425.35
1235411310	OUTREACH & COMMUNICA	Capital Outlay		$ 16,825.00		$ 16,825.00	$ -	$ -		-$ 16,825.00
1235411340	GENETIC MANIPULATION	All Budget Commitmen	$ 30,000.00	$ 7,797.18	$ 4,138.90	$ 11,936.08	$ 2,450.37	$ 15,473.06		$ 140.49
1235411340	GENETIC MANIPULATION	All Expenses Commitm	$ 30,000.00	$ 7,797.18	$ 4,138.90	$ 11,936.08	$ 2,450.37	$ 15,473.06		$ 140.49
1235411340	GENETIC MANIPULATION	TTL Expense Excl Tra	$ 30,000.00	$ 7,797.18	$ 4,138.90	$ 11,936.08	$ 2,450.37	$ 15,473.06		$ 140.49
1235411340	GENETIC MANIPULATION	Staff		$ 1,199.49	$ 630.49	$ 1,829.98	$ 1,891.47			-$ 3,721.45
1235411340	GENETIC MANIPULATION	Other Personnel		$ 3,772.29	$ 2,520.93	$ 6,293.22				-$ 6,293.22
1235411340	GENETIC MANIPULATION	Fringe Benefits		$ 271.72	$ 389.99	$ 661.71	$ 558.90			-$ 1,220.61
1235411340	GENETIC MANIPULATION	Operating Expense	$ 30,000.00	$ 2,553.68	$ 597.49	$ 3,151.17	$ -	$ 15,473.06		$ 11,375.77

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER

KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT

FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235411360	PLANT BIOTECH METABO	All Budget Commitmen	$ 30,000.00	$ 5,434.09	$ 2,717.03	$ 8,151.12	$ 24,664.23			-$ 2,815.35
1235411360	PLANT BIOTECH METABO	All Expenses Commitm	$ 30,000.00	$ 5,434.09	$ 2,717.03	$ 8,151.12	$ 24,664.23			-$ 2,815.35
1235411360	PLANT BIOTECH METABO	TTL Expense Excl Tra	$ 30,000.00	$ 5,434.09	$ 2,717.03	$ 8,151.12	$ 24,664.23			-$ 2,815.35
1235411360	PLANT BIOTECH METABO	Staff		$ 4,216.58	$ 2,108.29	$ 6,324.87	$ 18,974.61			-$ 25,299.48
1235411360	PLANT BIOTECH METABO	Fringe Benefits		$ 1,217.51	$ 608.74	$ 1,826.25	$ 5,689.62			-$ 7,515.87
1235411360	PLANT BIOTECH METABO	Operating Expense	$ 30,000.00							$ 30,000.00
1235411370	PLANT BIOTECH MOLECU	All Budget Commitmen	$ 33,487.00	$ 13,335.38	-$ 1,150.83	$ 12,184.55	$ -	$ -	$ -	$ 21,302.45
1235411370	PLANT BIOTECH MOLECU	All Expenses Commitm	$ 33,487.00	$ 13,335.38	-$ 1,150.83	$ 12,184.55	$ -	$ -	$ -	$ 21,302.45
1235411370	PLANT BIOTECH MOLECU	TTL Expense Excl Tra	$ 33,487.00	$ 13,335.38	-$ 1,150.83	$ 12,184.55	$ -	$ -	$ -	$ 21,302.45
1235411370	PLANT BIOTECH MOLECU	Other Personnel		$ 8,163.60	-$ 1,251.60	$ 6,912.00				-$ 6,912.00
1235411370	PLANT BIOTECH MOLECU	Fringe Benefits		$ 1,670.55	-$ 1,086.91	$ 583.64				-$ 583.64
1235411370	PLANT BIOTECH MOLECU	Operating Expense	$ 33,487.00	$ 3,501.23	$ 1,187.68	$ 4,688.91	$ -	$ -	$ -	$ 28,798.09
1235411410	GREENHOUSE	All Budget Commitmen	$ 42,604.00	$ 13,351.91	$ 726.45	$ 14,078.36	$ 4,696.00	$ 252.11	$ 44.72	$ 23,532.81
1235411410	GREENHOUSE	All Expenses Commitm	$ 42,604.00	$ 13,351.91	$ 726.45	$ 14,078.36	$ 4,696.00	$ 252.11	$ 44.72	$ 23,532.81
1235411410	GREENHOUSE	TTL Expense Excl Tra	$ 42,604.00	$ 13,351.91	$ 726.45	$ 14,078.36	$ 4,696.00	$ 252.11	$ 44.72	$ 23,532.81
1235411410	GREENHOUSE	Other Personnel		$ 2,376.48		$ 2,376.48				-$ 2,376.48
1235411410	GREENHOUSE	Operating Expense	$ 42,604.00	$ 10,975.43	$ 726.45	$ 11,701.88	$ 4,696.00	$ 252.11	$ 44.72	$ 25,909.29
1235411430	PLANT ANALYTIC	All Budget Commitmen	$ 17,478.00							$ 17,478.00
1235411430	PLANT ANALYTIC	All Expenses Commitm	$ 17,478.00							$ 17,478.00
1235411430	PLANT ANALYTIC	TTL Expense Excl Tra	$ 17,478.00							$ 17,478.00
1235411430	PLANT ANALYTIC	Operating Expense	$ 17,478.00							$ 17,478.00
1235411570	TOBACCO MOLECULAR	All Budget Commitmen		$ -	$ 642.96	$ 642.96			$ -	-$ 642.96
1235411570	TOBACCO MOLECULAR	All Expenses Commitm		$ -	$ 642.96	$ 642.96			$ -	-$ 642.96
1235411570	TOBACCO MOLECULAR	TTL Expense Excl Tra		$ -	$ 642.96	$ 642.96			$ -	-$ 642.96
1235411570	TOBACCO MOLECULAR	Operating Expense		$ -	$ 642.96	$ 642.96			$ -	-$ 642.96

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER

KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT

FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235411640	GENE DISCOVERY	All Budget Commitmen	$ 34,741.00	$ 12,033.06	-$ 1,665.79	$ 10,367.27	$ 47,477.57		$ -	-$ 23,103.84
1235411640	GENE DISCOVERY	All Expenses Commitm	$ 34,741.00	$ 12,033.06	-$ 1,665.79	$ 10,367.27	$ 47,477.57		$ -	-$ 23,103.84
1235411640	GENE DISCOVERY	TTL Expense Excl Tra	$ 34,741.00	$ 12,033.06	-$ 1,665.79	$ 10,367.27	$ 47,477.57		$ -	-$ 23,103.84
1235411640	GENE DISCOVERY	Staff		$ 8,115.84	-$ 1,310.73	$ 6,805.11	$ 34,731.74			-$ 41,536.85
1235411640	GENE DISCOVERY	Fringe Benefits		$ 2,938.55	-$ 475.06	$ 2,463.49	$ 12,745.83			-$ 15,209.32
1235411640	GENE DISCOVERY	Operating Expense	$ 34,741.00	$ 978.67	$ 120.00	$ 1,098.67	$ -		$ -	$ 33,642.33
1235412360	FLAVONOID - SMALLE	All Budget Commitmen	$ 30,715.00	$ 318.00	$ 5,441.80	$ 5,759.80	$ -	$ -	$ -	$ 24,955.20
1235412360	FLAVONOID - SMALLE	All Expenses Commitm	$ 30,715.00	$ 318.00	$ 5,441.80	$ 5,759.80	$ -	$ -	$ -	$ 24,955.20
1235412360	FLAVONOID - SMALLE	TTL Expense Excl Tra	$ 30,715.00	$ 318.00	$ 5,441.80	$ 5,759.80	$ -	$ -	$ -	$ 24,955.20
1235412360	FLAVONOID - SMALLE	Operating Expense	$ 30,715.00	$ 318.00	$ 5,441.80	$ 5,759.80	$ -	$ -	$ -	$ 24,955.20
1235412920	MUNDELL: TOBACCO HYB	All Budget Commitmen		$ 5,447.62	$ 2,230.51	$ 7,678.13				-$ 7,678.13
1235412920	MUNDELL: TOBACCO HYB	All Expenses Commitm		$ 5,447.62	$ 2,230.51	$ 7,678.13				-$ 7,678.13
1235412920	MUNDELL: TOBACCO HYB	TTL Expense Excl Tra		$ 5,447.62	$ 2,230.51	$ 7,678.13				-$ 7,678.13
1235412920	MUNDELL: TOBACCO HYB	Other Personnel		$ 5,030.00	$ 2,215.00	$ 7,245.00				-$ 7,245.00
1235412920	MUNDELL: TOBACCO HYB	Fringe Benefits		$ 417.62	$ 15.51	$ 433.13				-$ 433.13
1235412930	PATTANAIK: MALEIC HY	All Budget Commitmen					$ 1,523.98			-$ 1,523.98
1235412930	PATTANAIK: MALEIC HY	All Expenses Commitm					$ 1,523.98			-$ 1,523.98
1235412930	PATTANAIK: MALEIC HY	TTL Expense Excl Tra					$ 1,523.98			-$ 1,523.98
1235412930	PATTANAIK: MALEIC HY	Operating Expense					$ 1,523.98			-$ 1,523.98
1235412970	GOFF: OPTIMIZING INT	All Budget Commitmen		-$ 3.42		-$ 3.42				$ 3.42
1235412970	GOFF: OPTIMIZING INT	All Expenses Commitm		-$ 3.42		-$ 3.42				$ 3.42
1235412970	GOFF: OPTIMIZING INT	TTL Expense Excl Tra		-$ 3.42		-$ 3.42				$ 3.42
1235412970	GOFF: OPTIMIZING INT	Other Personnel		$ 25.00		$ 25.00				-$ 25.00
1235412970	GOFF: OPTIMIZING INT	Fringe Benefits		-$ 28.42		-$ 28.42				$ 28.42

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER
KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT
FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235413020	SMALLE: STRIGOLACTON	All Budget Commitmen		$ 37.74		$ 37.74		$ 39.85		-$ 77.59
1235413020	SMALLE: STRIGOLACTON	All Expenses Commitm		$ 37.74		$ 37.74		$ 39.85		-$ 77.59
1235413020	SMALLE: STRIGOLACTON	TTL Expense Excl Tra		$ 37.74		$ 37.74		$ 39.85		-$ 77.59
1235413020	SMALLE: STRIGOLACTON	Operating Expense		$ 37.74		$ 37.74		$ 39.85		-$ 77.59
1235413050	JACK LI LINES	All Budget Commitmen	$ 3,000.00	$ 70.22		$ 70.22			$ -	$ 2,929.78
1235413050	JACK LI LINES	All Expenses Commitm	$ 3,000.00	$ 70.22		$ 70.22			$ -	$ 2,929.78
1235413050	JACK LI LINES	TTL Expense Excl Tra	$ 3,000.00	$ 70.22		$ 70.22			$ -	$ 2,929.78
1235413050	JACK LI LINES	Operating Expense	$ 3,000.00	$ 70.22		$ 70.22			$ -	$ 2,929.78
1235413060	JACK STICK SPACING	All Budget Commitmen	$ 1,258.00	$ 72.72		$ 72.72			$ -	$ 1,185.28
1235413060	JACK STICK SPACING	All Expenses Commitm	$ 1,258.00	$ 72.72		$ 72.72			$ -	$ 1,185.28
1235413060	JACK STICK SPACING	TTL Expense Excl Tra	$ 1,258.00	$ 72.72		$ 72.72			$ -	$ 1,185.28
1235413060	JACK STICK SPACING	Operating Expense	$ 1,258.00	$ 72.72		$ 72.72			$ -	$ 1,185.28
1235413070	JI-ENANTIOMERS	All Budget Commitmen	$ 5,359.00	$ 2,758.65	$ 948.22	$ 3,706.87	$ 2,715.10	$ -	$ -	-$ 1,062.97
1235413070	JI-ENANTIOMERS	All Expenses Commitm	$ 5,359.00	$ 2,758.65	$ 948.22	$ 3,706.87	$ 2,715.10	$ -	$ -	-$ 1,062.97
1235413070	JI-ENANTIOMERS	TTL Expense Excl Tra	$ 5,359.00	$ 2,758.65	$ 948.22	$ 3,706.87	$ 2,715.10	$ -	$ -	-$ 1,062.97
1235413070	JI-ENANTIOMERS	Staff		$ 811.06	$ 405.53	$ 1,216.59	$ 2,027.65			-$ 3,244.24
1235413070	JI-ENANTIOMERS	Fringe Benefits		$ 265.72	$ 132.86	$ 398.58	$ 687.45			-$ 1,086.03
1235413070	JI-ENANTIOMERS	Operating Expense	$ 5,359.00	$ 1,681.87	$ 409.83	$ 2,091.70	$ -	$ -	$ -	$ 3,267.30
1235413080	PERR INCREASED TAG	All Budget Commitmen	$ 8,000.00							$ 8,000.00
1235413080	PERR INCREASED TAG	All Expenses Commitm	$ 8,000.00							$ 8,000.00
1235413080	PERR INCREASED TAG	TTL Expense Excl Tra	$ 8,000.00							$ 8,000.00
1235413080	PERR INCREASED TAG	Operating Expense	$ 8,000.00							$ 8,000.00

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER
KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT
FISCAL YEAR 2017 - 2018

Funds Center		Commitment item	Annual Budget	Prior Balance	Current Month Actual	YTD Actual	YTD Fund reser vations, reqs	YTD Encumbrances for PO's	YTD Parked FI docs	Available Budget
1235413090	KROU SCLAREOL BS SUP	All Budget Commitmen	$ 2,393.00		$ 1,473.68	$ 1,473.68				$ 919.32
1235413090	KROU SCLAREOL BS SUP	All Expenses Commitm	$ 2,393.00		$ 1,473.68	$ 1,473.68				$ 919.32
1235413090	KROU SCLAREOL BS SUP	TTL Expense Excl Tra	$ 2,393.00		$ 1,473.68	$ 1,473.68				$ 919.32
1235413090	KROU SCLAREOL BS SUP	Staff			$ 994.14	$ 994.14				-$ 994.14
1235413090	KROU SCLAREOL BS SUP	Other Personnel			$ 97.60	$ 97.60				-$ 97.60
1235413090	KROU SCLAREOL BS SUP	Fringe Benefits			$ 381.94	$ 381.94				-$ 381.94
1235413090	KROU SCLAREOL BS SUP	Operating Expense	$ 2,393.00							$ 2,393.00
1235413100	KROU CEMBR KNOCKDOWN	All Budget Commitmen	$ 6,000.00		$ 100.04	$ 100.04			$ 22.00	$ 5,877.96
1235413100	KROU CEMBR KNOCKDOWN	All Expenses Commitm	$ 6,000.00		$ 100.04	$ 100.04			$ 22.00	$ 5,877.96
1235413100	KROU CEMBR KNOCKDOWN	TTL Expense Excl Tra	$ 6,000.00		$ 100.04	$ 100.04			$ 22.00	$ 5,877.96
1235413100	KROU CEMBR KNOCKDOWN	Other Personnel			$ 100.04	$ 100.04				-$ 100.04
1235413100	KROU CEMBR KNOCKDOWN	Operating Expense	$ 6,000.00						$ 22.00	$ 5,978.00
1235413110	FISH BARN VENT	All Budget Commitmen	$ 404.00	$ 403.54		$ 403.54			$ -	$ 0.46
1235413110	FISH BARN VENT	All Expenses Commitm	$ 404.00	$ 403.54		$ 403.54			$ -	$ 0.46
1235413110	FISH BARN VENT	TTL Expense Excl Tra	$ 404.00	$ 403.54		$ 403.54			$ -	$ 0.46
1235413110	FISH BARN VENT	Operating Expense	$ 404.00	$ 403.54		$ 403.54			$ -	$ 0.46
1235413120	MILL EARLY BS VARIET	All Budget Commitmen	$ 12,000.00							$ 12,000.00
1235413120	MILL EARLY BS VARIET	All Expenses Commitm	$ 12,000.00							$ 12,000.00
1235413120	MILL EARLY BS VARIET	TTL Expense Excl Tra	$ 12,000.00							$ 12,000.00
1235413120	MILL EARLY BS VARIET	Operating Expense	$ 12,000.00							$ 12,000.00
1235413130	YANG AXILLARY BUD	All Budget Commitmen	$ 5,142.00	$ 3,250.50	$ 1,625.25	$ 4,875.75				$ 266.25
1235413130	YANG AXILLARY BUD	All Expenses Commitm	$ 5,142.00	$ 3,250.50	$ 1,625.25	$ 4,875.75				$ 266.25
1235413130	YANG AXILLARY BUD	TTL Expense Excl Tra	$ 5,142.00	$ 3,250.50	$ 1,625.25	$ 4,875.75				$ 266.25
1235413130	YANG AXILLARY BUD	Other Personnel		$ 3,000.00	$ 1,500.00	$ 4,500.00				-$ 4,500.00
1235413130	YANG AXILLARY BUD	Fringe Benefits		$ 250.50	$ 125.25	$ 375.75				-$ 375.75
1235413130	YANG AXILLARY BUD	Operating Expense	$ 5,142.00							$ 5,142.00

JULY 2017 - SEPTEMBER 2017

REPORTING 1ST QUARTER
KTRDC QUARTERLY REPORT

KTRDC FINANCIAL REPORT
FISCAL YEAR 2017 - 2018

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1235413140	YANG ULTRALOW ALKALO	All Budget Commitmen	$ 8,000.00							$ 8,000.00
1235413140	YANG ULTRALOW ALKALO	All Expenses Commitm	$ 8,000.00							$ 8,000.00
1235413140	YANG ULTRALOW ALKALO	TTL Expense Excl Tra	$ 8,000.00							$ 8,000.00
1235413140	YANG ULTRALOW ALKALO	Operating Expense	$ 8,000.00							$ 8,000.00
1235413150	WILL INDUSTRIAL HEMP	All Budget Commitmen	$ 3,852.00	$ 2,883.14		$ 2,883.14			$ -	$ 968.86
1235413150	WILL INDUSTRIAL HEMP	All Expenses Commitm	$ 3,852.00	$ 2,883.14		$ 2,883.14			$ -	$ 968.86
1235413150	WILL INDUSTRIAL HEMP	TTL Expense Excl Tra	$ 3,852.00	$ 2,883.14		$ 2,883.14			$ -	$ 968.86
1235413150	WILL INDUSTRIAL HEMP	Operating Expense	$ 3,852.00	$ 2,883.14		$ 2,883.14			$ -	$ 968.86
1235413160	KACH TRICH INDUCED	All Budget Commitmen	$ 11,661.00	$ 4,384.90	$ 3,482.57	$ 7,867.47	$ -	$ 321.34	$ -	$ 3,472.19
1235413160	KACH TRICH INDUCED	All Expenses Commitm	$ 11,661.00	$ 4,384.90	$ 3,482.57	$ 7,867.47	$ -	$ 321.34	$ -	$ 3,472.19
1235413160	KACH TRICH INDUCED	TTL Expense Excl Tra	$ 11,661.00	$ 4,384.90	$ 3,482.57	$ 7,867.47	$ -	$ 321.34	$ -	$ 3,472.19
1235413160	KACH TRICH INDUCED	Operating Expense	$ 11,661.00	$ 4,384.90	$ 3,482.57	$ 7,867.47	$ -	$ 321.34	$ -	$ 3,472.19
1235413170	PFEU FROGEYE MGMT	All Budget Commitmen	$ 12,000.00				$ 136.96			$ 11,863.04
1235413170	PFEU FROGEYE MGMT	All Expenses Commitm	$ 12,000.00				$ 136.96			$ 11,863.04
1235413170	PFEU FROGEYE MGMT	TTL Expense Excl Tra	$ 12,000.00				$ 136.96			$ 11,863.04
1235413170	PFEU FROGEYE MGMT	Operating Expense	$ 12,000.00				$ 136.96			$ 11,863.04

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