SEÑORES D.
DIRECCIÓN DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

EXPEDIENTE No. : NC2017/0004974
ASUNTO : SOLICITUD DE PATENTE DE INVENCIÓN TITULADA "**EXTRACTOS DE CANNABIS Y MÉTODOS DE PREPARACIÓN**".
SOLICITANTE : UNITED CANNABIS CORP.

Yo, **FERNANDO TRIANA SOTO**, ciudadano colombiano, mayor de edad, abogado en ejercicio y debidamente identificado como aparece al pie de mi firma, en mi calidad de apoderado de la sociedad **UNITED CANNABIS CORP.** existente y organizada de acuerdo con las leyes de los Estados Unidos de América, con domicilio en Denver, Colorado, Estados Unidos de América, de acuerdo con la prórroga de términos legales presentada el 19 de diciembre de 2018, me permito presentar respuesta al Oficio No. 11790, de acuerdo con las siguientes consideraciones:

1. DE LA SOLICITUD DE PATENTE

1.1 En relación a las reivindicaciones, el señor examinador expresa que las reivindicaciones 1 a 19 no son patentables, ya que al referirse a formulaciones donde el 95% o más del total de canabinoides en la formulación es ácido tetrahidrocanabinoico o tetrahidrocanabinol o canabidiol hace a la formulación indistinguible de los compuestos puros. Adicionalmente agrega que al caracterizar la formulación de manera que tiene terpenos como mirceno, cariofileno y limoneno no permite distinguir la formulación de los contenidos de metabolitos en la planta como se encuentran en la naturaleza, respecto a lo cual, el solicitante respetuosamente manifiesta que no está de acuerdo.

No obstante y con el ánimo de coadyuvar en el trámite que surte ese despacho, la reivindicación 1 es modificada en este documento para recitar "Una formulación *líquida* de cannabinoides, en donde la formulación *líquida* de cannabinoides es seleccionada desde un grupo que comprende: a. una formulación en la que el 95% o más de los cannabinoides totales en la formulación es ácido tetrahidrocannabinólico (THCa); b. una formulación en donde el 95% o más del total de cannabinoides en la formulación es tetrahidrocannabinol (THC); c. una formulación en la que el 95% o más del total de cannabinoides en la formulación es cannabidiol (CBD); d. una formulación en la que el 95% o más de los cannabinoides totales en la formulación son THCa y ácido cannabidiolico (CBDa); e. una formulación en la que el 95% o más de los cannabinoides totales en la formulación son THC y CBD; y f. una formulación en la que el 95% o más de los cannabinoides totales en la formulación son CBD, cannabinol (CBN) y THC" (*énfasis* agregado).

Todas las demás reivindicaciones sujetas al requerimiento dependen adecuadamente de la reivindicación 1 y, por lo tanto, incluyen todos los elementos de la reivindicación 1. La reivindicación 1, según se modificó en el presente documento, requiere que la formulación de cannabinoides sea un ***líquido***. Los cannabinoides en la naturaleza, es decir, en una planta de cannabis, son bien conocidos en la técnica por estar concentrados en una ***resina*** viscosa producida en estructuras conocidas como tricomas glandulares (ver, por ejemplo, Aizpurua-Olaizola y otros (2016) J. Natural Products, 79 (2): 324-331 y Booth, JK et al. (2017) PLoS ONE 12 (3):

e0173911; copias de cortesía proporcionadas a continuación). En química de polímeros y ciencia de materiales, una resina es "una substancia sólida suave o altamente viscosa, que generalmente contiene pre-polímeros con grupos reactivos" (http://goldbook.iupac.org/RT07166.html; copia de cortesía que es aquí proporcionada). Por lo tanto, la reivindicación 1, como es aquí enmendada, la cual recita una formulación líquida, incluye una característica marcadamente diferente cuando se compara con su producto natural. Esta característica marcadamente diferente da como resultado una distinción de las formulaciones de cannabinoides reivindicadas de sus contrapartes naturales que es relevante para la naturaleza de la invención como una substancia farmacológica botánica. Por ejemplo, las formulaciones reivindicadas pueden ser fácilmente formuladas para la administración oral, sublingual o tópica.

Por todas las razones anteriores, el Solicitante sostiene que la reivindicación enmendada 1 está dirigida a la materia elegible para la patente y el requerimiento en virtud del Artículo 15, literal b) es superada. De igual forma, ya que las reivindicaciones 2 a 19 dependen adecuadamente de la reivindicación 1, las reivindicaciones 2 a 19 también están dirigidas a la materia basada en la cláusula de la que dependen. Consecuentemente, se solicita respetuosamente reconsiderar y levantar las objeciones de la presente solicitud.

1.2 El examinador también advierte que el objeto de las reivindicaciones 37 y 38 no se consideran una invención, ya que es un líquido obtenido de la planta de cannabis tal y como se encuentra en la naturaleza. En respuesta a tal requerimiento, las reivindicaciones 37 y 38 son canceladas.

1.3 El examinador señala que las reivindicaciones 20 y 21 no son claras porque no se definen las condiciones bajo las cuales se llevan a cabo las etapas que conforman el método reclamado, de forma que no es posible limitar el alcance de la materia reclamada. Adelante define que las reivindicaciones 22, 23 y 28 no son claras, ya que el término “alrededor” no permite limitar el alcance de la invención, se sugiere definir la característica en un rango o valor específico. También define el examinador que la reivindicación 23 no tiene sustento en la descripción, ya que luego de hacer descarboxilación no se hace un calentamiento a 284°F, previamente se hace una infusión en aceite, y sugiere restringir la invención a los métodos que han sido elaborados. Las reivindicaciones 20 a 23 y 28 son rechazadas en virtud del artículo 30 porque supuestamente carecen de claridad. El solicitante respetuosamente manifiesta no estar de acuerdo.

El Oficio afirma que las reivindicaciones 20 y 21 no son claras, ya que no definen las condiciones bajo las cuales se llevan a cabo las etapas que conforman el método reivindicado. La reivindicación 21 es cancelada en este documento sin perjuicio ni exención de responsabilidad, por lo que se salva la objeción sobre esta reivindicación. Sin estar de acuerdo con las afirmaciones del Oficio y únicamente para acelerar el proceso, la reivindicación 20 es modificada como se indica previamente. La reivindicación 20, tal como es modificada en este documento, recita "*las condiciones o características de cada etapa del método*" tal como lo sugiere el Oficio. Por lo tanto, la cláusula 20, según se modifica en el presente documento, cumple con el Artículo 30 tal como lo exige el Oficio. Así, se pide reconsiderar y retirar el rechazo sobre esta materia.

Las reivindicaciones 22, 23 y 28 son rechazadas porque recitan la frase "alrededor de" que el Oficio "consideraba ambigua y no permitiría limitar el alcance de la invención". Sin conformarse con las afirmaciones del Oficio y únicamente para acelerar el proceso, las reivindicaciones 22, 23 y 28 se modifican en este documento para eliminar la frase "alrededor de". Así, el solicitante afirma que las reivindicaciones enmendadas 22, 23 y 28 cumplen con el Artículo 30 según lo exige el Oficio. Amablemente, pedimos reconsiderar y retirar la objeción sobre esta materia.

1.4 Así, con el ánimo de coadyuvar en la precisión y claridad de la invención, se presenta un nuevo pliego reivindicatorio, donde las reivindicaciones 1, 4, 9, 11, 14, 15, 17, 20, 22, 29 y 32 son aquí enmendadas. La reivindicación 1 es enmendada para incluir elementos de la reivindicación 2. La reivindicación 20 es enmendada para incluir elementos de las reivindicaciones 23, 27 y 28. La reivindicación 22 es enmendada para incluir elementos de la reivindicación 21. Las dependencias de las reivindicaciones 9, 11, 14, 15, 17 y 29 son modificadas para cumplir con las reivindicaciones reenumeradas. Las reivindicaciones 4, 15 y 32 son modificadas para corregir errores tipográficos. La reivindicación 18, es vuelta a numerar y es aquí agregada, es nueva. El apoyo para estas enmiendas y la nueva reivindicación puede ser encontrado en la Descripción y las reivindicaciones, tal como fueron presentadas originalmente, y a lo menos en [0063], [0076] - [0077] y [0098] de la memoria descriptiva presentada. Las reivindicaciones 2, 21, 23, 27-28 y 34-38 son aquí canceladas sin perjuicio, ni exención de responsabilidad. Es de aclarar que las nuevas reivindicaciones corregidas con base en lo solicitado no amplían la materia inicialmente radicada sino que hacen referencia a aclarar la presente invención conforme lo recomendado por el señor examinador.

2. UNIDAD DE INVENCIÓN

2.1 El señor examinador, procede también a definir que la solicitud no cumple con el requisito de unidad de invención porque se refiere a 2 grupos inventivos:

> *1° Una formulación de cannabinoides y un método para preparar un extracto de cannabis. Reivindicaciones 1 a 33. (Divulgados en los documentos D1 y D2)*
> *2° Un método para preparar un jugo de cannabis. Reivindicaciones 34 a 36.*

En respuesta a dicho requerimiento, las reivindicaciones 34 a 36 ahora son canceladas sin perjuicio ni exención de responsabilidad. El solicitante sostiene que las reivindicaciones dependientes en su forma enmendada, están dirigidas a un solo grupo inventivo, el del Grupo 1, de acuerdo a lo requerido por el Oficio.

3. RESPECTO AL ESTADO DE LA TÉCNICA

3.1 El señor examinador, procede a determinar el examen del estado de la técnica con base en los documentos D1: US8846409 y el documento D2: US2013/0059018. En ese sentido procede a establecer una tabla de comparación y concluye que la presente no comprende ni novedad ni altura inventiva frente a D1 y D2, de acuerdo con lo siguiente:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US8846409	20, 21, 24, 26, 27, 28 y 30	Novedad
		22, 23, 25, 29 y 31 a 33	Nivel inventivo
D2	US2013/0059018	20, 21, 24, 26, 27 y 28	Nivel inventivo

3.2 El documento D1 divulga métodos para preparar un extracto de cannabis que incluye descarboxilar, extraer los canabinoides y retirar el material ceroso (Col. 17).

3.3 El documento D2 divulga métodos para preparar un extracto de cannabis que incluye descarboxilar, extraer los canabinoides y retirar el material ceroso (Fig. 1).

3.4 Las reivindicaciones 20, 21, 24, 26-28 y 30 son rechazadas porque supuestamente carecen de novedad frente a **D1** (US8846409). El solicitante respetuosamente no está de acuerdo. Sin aceptar las afirmaciones del Oficio y únicamente para acelerar el proceso de la solicitud, la reivindicación 20 es modificada con el presente para recitar:

"Un método para preparar un extracto de cannabis donde el método comprende: a. proveer material de plantas de cannabis vivas o frescas; b. extraer los cannabinoides desde el material de plantas para producir un primer extracto, en el donde la extracción comprende una extracción con hidrocarburo, una extracción supercrítica con CO_2 o una extracción con NEOBEE 895; c. congelar el extracto de la etapa (b) para producir un extracto almacenado, en donde congelar comprende agregar etanol frío al primer extracto o almacenar el primer extracto a una temperatura de -28,8°C a -59,4°C (-20º a -75º F) durante 48 horas para producir un precipitado ceroso y eliminar el precipitado ceroso por filtración; d. purgar el extracto congelado de la etapa (c) al vacío para producir un extracto de cannabis; y e. calentar el extracto de la etapa (d) a 140°C (284 °F) durante 45-74 minutos, seguido de calentamiento a 145°C (293 °F) durante al menos 55-90 minutos".

La reivindicación 20, en su versión enmendada, indica *"e. calentar el extracto de la etapa (d) a 140°C (284 °F) durante 45-74 minutos, seguido de calentado a 145°C (293 °F) durante a lo menos 55-90 minutos"*. D1 falla en describir o sugerir un método para preparar un extracto de cannabis que comprenda *" e. calentar el extracto de la etapa (d) a 140°C (284°F) durante 45-74 minutos, seguido de calentado a 145°C (293 °F) durante a lo menos 55-90 minutos*", según lo exige la reivindicación 20 enmendada. Ya que **D1** falla en describir o sugiere todos los elementos de la reivindicación 20, la reivindicación 20 es nueva sobre **D1**.

3.5 Las reivindicaciones 21, 24, 26-28 y 30 dependen de la reivindicación 20 y, por lo tanto, recitan todos los elementos de la reivindicación 20. Por lo tanto, las reivindicaciones 20, 21, 24, 26-28 y 30 son nuevas sobre **D1**. Ya que las reivindicaciones 20, 21, 24, 26-28 y 30 son nuevas sobre **D1**, la reivindicación 1 también es nueva sobre **D1**. Así, se solicita respetuosamente reconsiderar y levantar el rechazo sobre la presente solicitud.

3.6 Las reivindicaciones 20, 21, 24 y 26-28 son rechazadas debido a que supuestamente carecen de novedad sobre **D2** (US2013/0059018). El solicitante respetuosamente no está de acuerdo. Sin estar de acuerdo con las afirmaciones del Oficio y únicamente para acelerar el proceso de la prosecución de la presente patente, la reivindicación

20 es modificada como se indicó con anterioridad. Así, **D2** no describe ni sugiere una etapa de preparación acondicionada para el invierno "*en donde congelar comprende agregar etanol frío al primer extracto o almacenar el primer extracto a una temperatura de -28,8°C a -59,4°C (-20º a -75º F) durante 48 horas*" como lo exige la reivindicación 20. **D2** también falla en describir o sugerir "*e. calentar el extracto de la etapa (d) a 140°C (284 °F) durante 45-74 minutos, seguido de calentado a 145°C (293 °F) durante a lo menos 55-90 minutos*", como enseña la reivindicación 20. Por lo tanto, **D2** no describe o sugiere todos los elementos de la reivindicación 20 enmendada, y la reivindicación 20 es nueva sobre **D2**.

3.7 Las reivindicaciones 21, 24 y 26-28 dependen de la reivindicación 20 y, por lo tanto, recitan todos los elementos de la reivindicación 20. En tal razón, las reivindicaciones 20, 21, 24 y 26-28 son nuevas sobre **D2**. Debido a que las reivindicaciones 20, 21, 24 y 26-28 son nuevas sobre D2, la reivindicación 1 también es nueva sobre **D2**. En conclusión, se solicita respetuosamente la reconsideración y el retiro de la objeción.

3.8 Las reivindicaciones 22, 23, 25, 29 y 31-33 son rechazadas porque supuestamente carecen de un nivel inventivo frente a **D1**. El solicitante respetuosamente no está de acuerdo, no obstante, con el propósito de acelerar el proceso, la reivindicación 20 es modificada aquí como es anteriormente indicado. Las reivindicaciones 22, 23, 25, 29 y 31-33 dependen adecuadamente de la reivindicación 20 y, por lo tanto, recitan todos los elementos de la reivindicación 20.

3.9 Como ha sido previamente argumentado para la novedad, **D1** no describe o sugiere un método para preparar extracto de cannabis que comprenda *"e. calentar el extracto de la etapa (d) a 140°C (284°F) durante 45-74 minutos, seguido de calentado a 145°C (293 °F) durante a lo menos 55-90 minutos*", según lo exige la reivindicación 20 enmendada.

3.10 Con respecto a esta etapa, la memoria descriptiva, tal como se presenta, establece que *"[a] el aceite descarboxilado resultante de CBD: THC es convertido en aceite de CBD: CBN (definido como> 90% de CBD: THC) calentando el aceite a 140°C (284 °F) por 45- 75 minutos, y una segunda temperatura a 145°C (293 ° F) durante 55 minutos - 90 minutos"* (Párrafo [00105]). Por lo tanto, la diferencia entre la presente invención y los métodos de **D1** es la provisión de una etapa de calentado adicional que convierte el extracto de cannabis de las reivindicaciones dependientes en CBD: CBN. Según lo enseñado por la memoria descriptiva presentada, CBN es el producto principal de la degradación del THC, y en contraste con el THC, es sólo ligeramente psicoactivo (véase memoria descriptiva, Párrafos [0037] a [0039]). Por consiguiente, las composiciones producidas por los métodos de las reivindicaciones dependientes son menos psicoactivas que las composiciones de **D1**.

3.11 Por lo tanto, en vista de las ventajas previamente descritas, el problema técnico objetivo puede ser formulado como métodos ***mejorados*** de preparación de un extracto de cannabis que comprende CBD: CBN y no "cómo obtener métodos alternativos de lo que es conocido por **D2**" como afirma el Oficio.

3.12 La solución reivindicada al problema implica una etapa inventiva. Así, **D1** indica "Al seleccionar las condiciones apropiadas para la descarboxilación, se debe

considerar, sin embargo, la minimización de la degradación térmica de los cannabinoides farmacológicos deseables en productos de degradación indeseables, particularmente la degradación térmica de Δ^9 THC a cannabinol (CBN)" (**D1**, Columna 5, líneas 14-18). Por lo tanto, **D1** enseña que el THC es un cannabinoide farmacológicamente activo deseable, que el THC es degradado por temperaturas más altas en CBN, y que esta degradación es indeseable ya que reduce el THC farmacológicamente activo en el extracto.

3.13 Por lo tanto, el experto en la materia, en vista enseñanzas de **D1** y los conocimientos de la técnica en el momento de la presentación, no se vería motivado a modificar **D1** al agregar una etapa de calentamiento adicional para llegar a la invención de la reivindicación 20 con cualquier expectativa razonable de resultados predecibles o éxito. La reivindicación 20 es por consiguiente inventiva frente a **D1**. Debido a que las nuevas cláusulas 21 a 29 dependen en forma apropiada de la reivindicación 20, se describen todos los elementos de la reivindicación 20. Por lo tanto, ahora son inventivas sobre **D1**. La reconsideración y el retiro del rechazo se solicitan respetuosamente.

3.14 En ese sentido, se ha modificado el nuevo pliego aquí presentado, redactado con caracterizaciones que lo diferencian aun más de la materia expuesta en D1 y D2.

3.15 Como se ha modificado el pliego reivindicatorio en la medida que se ha definido el estado "líquido" dentro del preámbulo de las reivindicaciones 1 a 19 y se ha definido la reivindicación 20 y sus subsidiarias, habida cuenta que no se ha sobrepasado el periodo de 18 meses a partir de la publicación de la presente, **solicito formalmente se practique un nuevo examen de patentabilidad**. Para tal efecto anexo a la presente el pago respectivo, para que sea evaluado en términos de novedad y altura inventiva, tal como se reclama en el nuevo capítulo reivindicatorio para determinar que no está afectada por dichos documentos D1 y D2, ya que se ha probado que estos documentos no son perjudiciales respecto al requisito de ALTURA INVENTIVA o la NOVEDAD de la invención, ni contradice cualquier disposición legal o reglamentaria.

3.16 En consecuencia, a la luz de todo lo anterior, me permito solicitar a ese despacho, esta solicitud sea evaluada con un nuevo examen de patentabilidad y la misma sea concedida bajo el tenor de las nuevas reivindicaciones. Sin embargo, incluso si el examinador lo considera insuficiente en conjunto con las nuevas reivindicaciones o el razonamiento que aquí se presenta, la solicitante ruega respetuosamente que se le ofrezca una nueva oportunidad, a través un nuevo Oficio para expresarse sobre cualquier tema en controversia.

4. ANEXOS

4.1 Pago de un nuevo examen de patentabilidad.

4.2 Un nuevo capítulo reivindicatorio.

4.3 Copias de los siguientes documentos:

- *Aizpurua-Olaizola et al. (2016) J. Natural Products, 79(2): 324-331 and Booth, J.K. et al. (2017) PLoS ONE 12(3): e0173911.*
- *http://goldbook.iupac.org/RT07166.html;*
- *Judith K. Booth1, Jonathan E. Page2,3, JoÈrg Bohlmann1,3*, Terpene synthases from Cannabis sativa.*

5. NOTIFICACIONES

Para efectos de notificaciones que por disposición debe hacer su Despacho, informo que mi representada y el suscrito recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas de abogado situadas en la Calle 93B No. 12-48, Piso 4, de la ciudad de Bogotá.

Del Señor Director,

FERNANDO TRIANA SOTO
C.C. 79.154.036 de Bogotá
T.P.A. 45.265 del C. S. de la J.

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Colo 7804 0301 76755

REIVINDICACIONES

1. Una formulación líquida de canabinoides, en donde la formulación líquida de canabinoides se selecciona del grupo que consiste en:

    a. Una formulación donde el 95% o más del total de canabinoides en la formulación es ácido tetrahidrocanabinoico (THCa);
    b. Una formulación donde el 95% o más del total de canabinoides en la formulación es tetrahidrocanabinol (THC);
    c. Una formulación donde el 95% o más del total de canabinoides en la formulación es canabidiol (CBD);
    d. Una formulación donde el 95% o más del total de canabinoides en la formulación es THCa y ácido canabidiólico (CBDa);
    e. Una formulación donde el 95% o más del total de canabinoides en la formulación es THC y CBD; y
    f. Una formulación donde el 95% o más del total de canabinoides en la formulación es CBD, canabinol (CBN) y THC.

2. La formulación de canabinoides de la reivindicación 1, donde la formulación comprende además uno o más terpenos o flavonoides.
3. La formulación de canabinoides de la reivindicación 2, donde la formulación de canabinoides comprende no más del 4% de terpenos.
4. La formulación de canabinoides de la reivindicación 1, donde el 95% o más del total de canabinoides de la formulación es CBD y la formulación comprende menos de 1% de THC.
5. La formulación de canabinoides de la reivindicación 1, donde el 95% o más del total de canabinoides de la formulación son CBD, CBN y THC, y la formulación comprende menos del 9% de THC.
6. La formulación de canabinoides de la reivindicación 1, donde el 95% o más de los canabinoides de la formulación es THCa, y la formulación comprende no más de 4% en terpenos.
7. La formulación de canabinoides de la reivindicación 1, donde el 95% o más de los canabinoides de la formulación es THC, y la formulación comprende no más de 4% en terpenos.
8. La formulación de canabinoides de las reivindicaciones 6 ó 7, donde el terpeno comprende mirceno, cariofileno y limoneno.
9. La formulación de canabinoides de la reivindicación 1, donde el 95% o más de los canabinoides de la formulación es CBD, y la formulación comprende no más de 4% en terpenos.
10. La formulación de canabinoides de las reivindicación 9, donde el terpeno comprende mirceno, cariofileno y limoneno.
11. La formulación de canabinoides de la reivindicación 1, donde el 95% o más de los canabinoides de la formulación son THCa y CBDa, y la formulación comprende no más de 4% en terpenos.
12. La formulación de canabinoides de la reivindicación 1, donde el 95% o más de los canabinoides de la formulación son THC y CBD, y la formulación comprende no más de 4% en terpenos.
13. La formulación de canabinoides de las reivindicaciones 11 ó 12, donde el terpeno comprende limoneno, pineno y cariofileno.

14. La formulación de canabinoides de las reivindicaciones 11 ó 12, donde el terpeno comprende mirceno, limoneno, pineno y cariofileno.
15. La formulación de canabinoides de la reivindicación 1, donde el 95% o más de los canabinoides de la formulación son CBD, CBN y THC, y la formulación comprende no más de 4% en terpenos.
16. La formulación de canabinoides de las reivindicación 15, donde el terpeno comprende mirceno, pineno y cariofileno.
17. La formulación de canabinoides según cualquiera de las reivindicaciones precedentes, donde la formulación de canabinoides se infunde en un triglicérido de cadena mediana.
18. La formulación de canabinoides de las reivindicación 17, en la que el triglicérido de cadena media es NEOBEE 895 MCT.
19. La formulación de canabinoides según cualquiera de las reivindicaciones precedentes, donde el pH de la formulación de canabinoides es de 8,0 o más.
20. Un método para preparar un extracto de cannabis, donde el método comprende:
    a. Proveer material de plantas de cannabis vivas o frescas;
    b. Extraer los canabinoides desde el material de plantas para producir un primer extracto, en donde la extracción comprende una extracción de hidrocarburos, una extracción supercrítica de CO2 o una extracción NEOBEE 895;
    c. congelar el extracto del paso (b) para producir un extracto almacenado, en donde la preparación congelada comprende agregar etanol frío al primer extracto o almacenar el primer extracto a una temperatura de -28,889º (-20º) a -23,889 ºC (75º F) durante 48 horas para producir un precipitado ceroso;
    d. Purgar el extracto congelado del paso (c) al vacio para producir un extracto de cannabis; y,
    e. calentar el extracto de la etapa (d) a 29,536 ºC (284 °F) durante 45-74 minutos, seguido de calentamiento a 145 ºC (293 °F) durante al menos 55-90 minutos.
21. El método de la reivindicación 20, el que además comprende descarboxilar los fitocanabinoides antes del paso (b), y donde la descarboxilación comprende calentar las plantas secas a una temperatura de alrededor de 105ºC (221°F) durante 15 minutos o más, seguido por calentamiento a alrededor de 29,536 ºC (284 °F) durante 45 minutos o más.
22. El método de la reivindicación 20, donde el material de plantas consiste en flores o flores y hojas.
23. El método de la reivindicación 20, en donde el material de plantas de cannabis se congela a una temperatura entre -23,333ºC (-10°F) a -45,556ºC (-50°F), por 36 horas o más antes de ser extraídas.
24. El método de la reivindicación 20, el material de plantas de cannabis ha sido propagado desde una semilla única o un tejido cultivado con razones específicas de canabinoides.
25. El método de la reivindicación 20, el que además comprende filtrar el extracto congelado a través de carbón activado.
26. El método de la reivindicación 20, donde el material de planta de cannabis se deriva de una cepa que tiene un mínimo de 15% de THC y un mínimo de 1% de CBD.
27. El método de la reivindicación 20, donde el material de planta de cannabis se deriva de las cepas Sour Tsunami x Catatonic Sour Tsunami x Sour Tsunami, Sour Tsunami, Harlequin, R4 o ACDC.
28. El método de la reivindicación 20, donde el material de planta de cannabis se deriva de las cepas CBD1, Sour Pineapple, CBD Diesel, Harlequin, ACDC o R4.

29. El método de la reivindicación 21, donde el material de planta de cannabis se deriva de las cepas Sour Tsunami x Catatonic, Sour Tsunami x Sour Tsunami, Sour Tsunami, Harlequin, R4, Swiss Gold, ACDC, CBD1, Sour Pineapple o CBD Diesel.

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RESEARCH ARTICLE

Terpene synthases from *Cannabis sativa*

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Abstract

Cannabis (*Cannabis sativa*) plants produce and accumulate a terpene-rich resin in glandular trichomes, which are abundant on the surface of the female inflorescence. Bouquets of different monoterpenes and sesquiterpenes are important components of cannabis resin as they define some of the unique organoleptic properties and may also influence medicinal qualities of different cannabis strains and varieties. Transcriptome analysis of trichomes of the cannabis hemp variety `Finola' revealed sequences of all stages of terpene biosynthesis. Nine cannabis terpene synthases (CsTPS) were identified in subfamilies TPS-a and TPS-b. Functional characterization identified mono- and sesqui-TPS, whose products collectively comprise most of the terpenes of `Finola' resin, including major compounds such as β-myrcene, (*E*)-β-ocimene, (-)-limonene, (+)-α-pinene, β-caryophyllene, and α-humulene. Transcripts associated with terpene biosynthesis are highly expressed in trichomes compared to non-resin producing tissues. Knowledge of the CsTPS gene family may offer opportunities for selection and improvement of terpene profiles of interest in different cannabis strains and varieties.

Introduction

Cannabis sativa, referred to here as cannabis, has been used for millennia as a medicine and recreational intoxicant [1, 2]. The species *Cannabis sativa* comprises both marijuana and hemp [3, 4, 5]. Medicinal cannabis is highly valued for its pharmacologically active cannabinoids, a class of terpenophenolic metabolites unique to cannabis. These compounds are primarily found in the resin produced in the glandular trichomes of pistillate (female) inflorescences. Cannabis resin also contains a variety of monoterpenes and sesquiterpenes (Fig 1), which are responsible for much of the scent of cannabis flowers and contribute characteristically to the unique flavor qualities of cannabis products. Similarly, terpenes in hop (*Humulus lupulus*), a close relative of cannabis, are an important flavoring component in the brewing industry. Differences between the pharmaceutical properties of different cannabis strains have been attributed to interactions (or an `entourageeffect') between cannabinoids and terpenes [6, 7]. For example, the sesquiterpene β-caryophyllene interacts with mammalian cannabinoid receptors [8]. As a result, medicinal compositions have been proposed to incorporate blends of cannabinoids and terpenes [9]. Terpenes may contribute anxiolytic, antibacterial, anti-inflammatory, and sedative effects [6].

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Competing interests: JEP is the CEO and President of Anandia Labs Inc. JB is a consultant and adviser to CannaRoyalty Corp. (since December 2016). These affiliations do not alter our adherence to PLOS ONE policies on sharing data and materials.

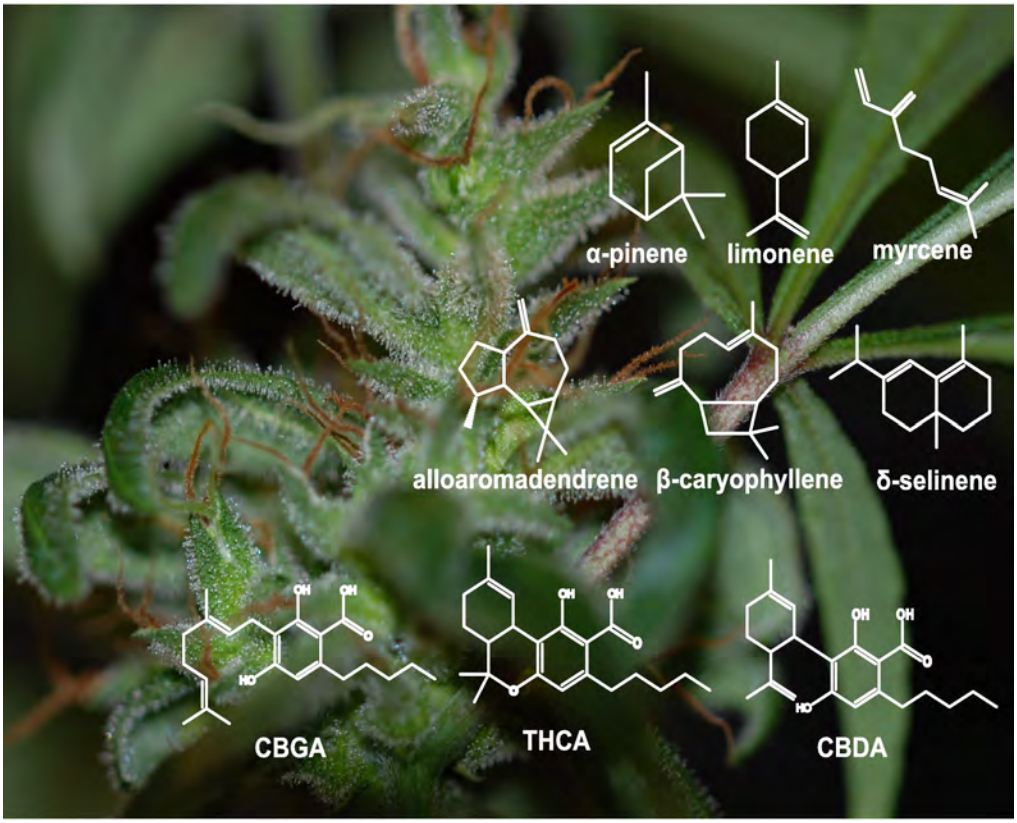

Fig 1. Glandular trichomes on the surface of pistillate inflorescences and leaves of *Cannabis sativa* `Finola'. The inflorescence (left) with a high density of glandular trichomes was at five weeks post onset of flowering. Non-inflorescence leaves (right) have lower density of glandular trichomes. Structures of representative cannabis resin components are shown in white: monoterpenes (top row), sesquiterpenes (middle row), and cannabinoids (bottom row). GBGA = cannabigerolic acid; THCA = tetrahydrocannabinolic acid; CBDA = cannabidiolic acid.

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Terpene biosynthesis in plants involves two pathways to produce the general 5-carbon isoprenoid diphosphate precursors of all terpenes, the plastidial methylerythritol phosphate (MEP) pathway and the cytosolic mevalonate (MEV) pathway. These pathways ultimately control the different substrate pools available for terpene synthases (TPS). The MEP pathway is comprised of seven steps that convert pyruvate and glyceraldehyde-3-phosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Fig 2A). Enzymes thought to be critical for flux regulation through this pathway include the first two and final two steps: 1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxy-D-xylulose 5-phosphate reductase, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase, and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase [10, 11]. The MEV pathway converts three units of acetyl-CoA to IPP, which is then isomerized to DMAPP by IPP isomerase. A rate-limiting step in this six-step pathway is 3-hydroxy-3-methylglutaryl-CoA reductase, which produces mevalonate [12]. IPP and DMAPP are condensed into longer-chain isoprenoid diphosphates by prenyltransferases, which include geranyl diphosphate (GPP) synthase (GPPS) and farnesyl diphosphate (FPP) synthase (FPPS). GPPS and FPPS condense one unit of IPP and one or two units of DMAPP to form 10- and 15-carbon linear *trans*-isoprenoid diphosphates, respectively. GPP is the 10-carbon precursor of monoterpenes and is typically derived from 5-carbon isoprenoid diphosphate units of the MEP pathway. GPP is also a building block in the biosynthesis of

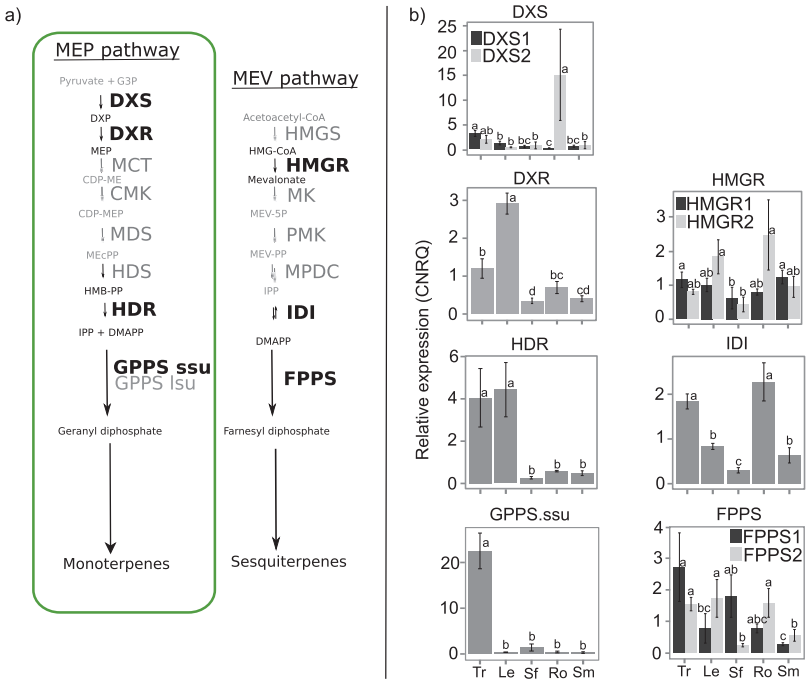

Fig 2. Schematic of the plastidial methylerythritol phosphate pathway (MEP) and mevalonic acid pathway (MEV) and transcript abundance in different parts of cannabis. Steps shown in bold (a) were included in the qPCR analysis (b) of relative abundance of transcripts. Letters indicate significantly different means between tissues (tested within each gene), Fisher's LSD (alpha = 0.05). Abbreviations: Tr = trichome; Le = leaf; Sf = stamenate flower; Ro = root; Sm = stem.

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cannabinoids [13, 14]. FPP is the 15-carbon precursor of sesquiterpenes and is commonly produced from 5-carbon isoprenoid diphosphate units of the cytosolic mevalonate (MEV) pathway. GPPSs exist as homo- or heterodimeric enzymes. In hops, the closest known relative of cannabis, heterodimeric GPPSs can produce both GPP and the 20-carbon geranylgeranyl diphosphate (GGPP), with the ratio of large to small G(G)PPS subunits controlling the product outcome [15, 16, 17]. The linear isoprenoid diphosphates GPP and FPP are substrates for monoterpene synthases (mono-TPS) and sesquiterpene synthases (sesqui-TPS), respectively, which diversify these precursors into a large number of different mono- and sesquiterpenes.

TPS are typically encoded in large and diverse gene families in plants [18], where they contribute to both general and specialized metabolism. The plant TPS gene family has been annotated with six subfamilies. In angiosperms, the subfamily TPS-b is typically comprised of mono-TPS and TPS-a enzymes are often sesquiterpene synthases. TPS produce cyclic and acyclic terpenes via carbocationic intermediates, formed by divalent metal co-factor dependent elimination of the diphosphate. The reactive cationic intermediate can undergo cyclization and rearrangements until the reaction is quenched by deprotonation or water capture [19]. Many TPS form multiple products from the same substrate.

The terpene composition of cannabis resin varies substantially based on genetic, environmental, and developmental factors [20, 21, 22, 23]. Concentrations and ratios of cannabinoids are relatively predictable for different strains, but terpene profiles are often unknown or unpredictable [20, 23]. To select and improve cannabis strains with desirable terpene profiles, it is necessary to identify genes responsible for terpene biosynthesis, which can be accomplished by harnessing available cannabis transcriptome and genome resources. Draft genomes and transcriptomes for the marijuana strain Purple Kush and the hemp variety `Finola'have previously

been published [24]. We used these resources to explore the expression of genes involved in all stages of terpene biosynthesis. We identified nine *TPS* gene models in the `Finola' transcriptome. *TPS* genes and gene transcripts in the MEP and MEV pathways were highly expressed in floral trichomes. We identified biochemical functions of *TPS* that are highly expressed in `Finola'. The TPS enzymes characterized account for most of the terpenes found in `Finola' resin.

Materials and methods

Plant materials

Cannabis seeds, `Finola', were obtained from Alberta Innovates Technology Futures (www.albertatechfutures.ca). All plants were grown indoors in a growth chamber under a Health Canada license. Seeds were germinated on filter paper, then transferred to 4:1 Sunshine Mix #4 (www.Sungro.com):perlite. Daylight length was 16 h under fluorescent lights, and ambient temperature 28ÊC. About two weeks after germination, seedlings were transferred to larger pots. After repotting, all plants were fertilized weekly with Miracle-Gro all-purpose plant food (24-8-26) (www.miraclegro.com) according to manufacturer's instructions.

Terpene extraction

Pistillate inflorescences were collected and trimmed of leaves and stems. All flowers from an individual plant were pooled. Tissue samples of ~0.2 g were weighed to determine fresh weight. Three rounds of extraction in 1 ml of pentane were performed for 1 hour each at room temperature with gentle shaking. Isobutyl benzene was added as an internal standard. After three extractions, no terpenes were identified in a fourth solvent extraction. Floral tissue was then dried overnight and weighed to determine dry weight. All three pentane extracts were combined for a total volume of 3 ml for analysis.

Trichome isolation

The heads of glandular trichomes were isolated from whole inflorescences as previously described [25] without XAD-4 and with the addition of 5 mM aurintricarboxilic acid in the isolation buffer. Instead of a cell disruptor, floral tissue was vortexed with glass beads in a Falcon tube to remove trichome heads. After vortexing, trichomes were separated from beads and green tissue by filtration through a 105 μm nylon mesh. Trichomes were concentrated by gentle centrifugation in ice-cold buffer. The supernatant was removed with a pipette, and the pellet of trichomes was immediately frozen in liquid nitrogen.

Metabolite analysis

Gas chromatography (GC) analysis of floral extracts was performed on an Agilent (www.chem.agilent.com) 7890A GC with a 7683B series autosampler and 7000A TripleQuad mass spectrometer (MS) detector at 70 eV electrospray ionization with a flow rate of 1 ml min^{-1} He. The column was an Agilent VF-5MS or DB-5MS (30 m, 250 μm internal diameter, 0.25 μm film). The following temperature program was used: 50ÊC, then increase 150ÊCmin^{-1} to 320ÊC, hold for 5 minutes. Injection was pulsed splitless at 250ÊC. Compounds were identified by comparison of retention index and mass spectra to authentic standards. Standards were available for all monoterpenes and the following sesquiterpenes: β-caryophyllene, α-humulene, farnesol, valencene, germacrene D, and alloaromadendrene. Tentative identifications for all other sesquiterpenes were made by comparison of retention index and mass spectra to National Institute of Standards and Technology (NIST) MS library. Identifications of

bergamotene, δ-selinene, and farnesene were strengthened by comparison to essential oils of *Citrus bergamia* (Bergamot) and *Pimenta racemose* (Bay) (www.lgbotanicals.com). TPS assay products were analyzed by the same procedure described above for plant extracts, but with the following temperature program: 50ÊCfor 3 minutes, then increase 15ÊCmin^{-1} to 280ÊC,hold for 2 minutes. Assay products were analyzed using Agilent HP-5 and DB-Wax columns (30 m length, 250 μm internal diameter, 0.25 μm film). For cold injection of sesqui-TPS assay products, the following program was used on a DB-Wax column: 40ÊCfor 3 minutes, then increase 10ÊCmin^{-1} to 230ÊC,hold for 7 minutes. Injection was at 40ÊCwith a 1:1 split ratio. Chiral analysis of terpenes was done using a Cyclodex-B column (30 m length, 250 μm internal diameter, 0.25 μm film). Injection was pulsed splitless, with the following program: 40ÊCfor 1 minute, then increase 5ÊCmin^{-1} to 100ÊC,then increase 15ÊCmin^{-1} to 250ÊC,hold for 4 minutes. Chirality was determined by retention index and comparison with authentic standards.

cDNA cloning and characterization of *TPS* genes

Total RNA was isolated from `Finola' flowers, leaves, stem, and roots using Invitrogen PureLink Plant RNA reagent (www.thermofisher.com). RNA quality and concentration was measured with a Bioanalyzer 2100 RNA Nanochip assay (www.agilent.ca). cDNA was synthesized with the Superscript III reverse transcriptase kit (Thermo Fisher). Full length and N-terminally truncated cDNAs without transit peptides where applicable [26] were amplified from cDNA using gene-specific primers (S1 Table) designed from published transcriptomic data [24]. N-terminal transit peptides were predicted based on sequence alignments [27] and using the TargetP and ChloroP servers [28]. PCR amplified `Finola' cDNAs were ligated into pJET vector (www.clontech.com) for sequence verification, and subcloned into expression vectors pET28b+ (www.endmillipore.ca) or pASK-IBA37 (www.iba-lifesciences.org) in the case of CsTPS5FN.

High-confidence full-length *TPS* cDNA candidates from Purple Kush (CsTPS13PK, CsTPS30PK, and CsTPS33PK) were synthesized by GenScript (www.genscript.com) into pET28b+. For this purpose, putative *TPS* sequences from Purple Kush transcriptome data were verified by comparison to genomic sequences [24].

Plasmids were transformed into *E. coli* strain BL21DE3-C43 for heterologous protein expression, as previously described [29]. Recombinant protein was purified using the GE healthcare His SpinTrap kit (www.gelifesciences.com) according to manufacturer's instructions. Binding buffer for purification was 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (pH 7.5), 500 mM NaCl, 25 mM imidazole, and 5% glycerol. Cells were lysed in binding buffer supplemented with Roche complete protease inhibitor tablets (lifescience.roche.com) and 0.1 mg ml^{-1} lysozyme. Elution buffer was 20 mM HEPES (pH 7.5), 500 mM NaCl, 350 mM imidazole, and 5% glycerol. Purified protein was desalted through Sephadex into TPS assay buffer. *In vitro* assays were performed in 500 μl volume by incubating purified protein with isoprenoid diphosphate substrates (Sigma) as previously described [30], except that the TPS assay buffer was 25 mM HEPES (pH 7.3), 100 mM KCl, 10 mM $MgCl_2$, 5% glycerol, and 5 mM DTT. Isoprenoid diphosphate substrates were dissolved in 50% methanol and added to the assay at final concentrations of 32 μM (GPP) and 26 μM (FPP). Enzyme concentrations were variable ranging from 20 to 100 μg per 500 μl assay volume. Assays were overlaid with 400 μl hexane or pentane, with 2.5 μM isobutyl benzene as internal standard.

Nicotiana benthamiana transformation and transient expression

The CsTPS5FN coding sequence was inserted into the Golden Gate plant expression vector pEAQ-GG, which contains a CaMV 35S promoter. This construct and the suppressor-of-silencing gene p19 were transformed into *Agrobacterium tumefasciens* strain AGL1. For

infiltration, *A. tumefasciens* was grown overnight as previously described [31], then pelleted and resuspended in 10 mM 2-(N-morpholine)-ethanesulphonic acid (MES) buffer, pH 5.8, 10 mM $MgCl_2$, 20 μM acetosyringone to OD_{600} 0.5. Equal volumes of bacteria, 25 ml each, containing TPS5 and p19 were infiltrated into the abaxial side of 4-week-old *N. benthamiana* plants. Infiltrated plants were grown for three days in the dark. Infiltrated leaves were harvested and ground in TPS assay buffer, and enzyme activity assays were conducted as above.

RT-qPCR analysis of transcript abundance

cDNA for qPCR was synthesized using the Maxima First Strand cDNA synthesis kit (Thermo Fisher) according to manufacturer's instructions. qPCR reactions were done in 15 μl volumes with SsoFast EvaGreen supermix (Bio-Rad), 4 μl template (2 ng), and 0.3 μM primers. Primers (S2 Table) were designed using Primer3 software [32]. Reference genes were chosen by geNorm [33], analyzed with qBase+ software (www.biogazelle.com). Reference genes used for RT-qPCR of early isoprenoid biosynthesis across different plant organs were *actin* and *CDK3*. For RT-qPCR of *TPS* transcripts in trichomes, reference genes were *CDK3* and *GAPDH*. RT-qPCR analyses were done with four biological and two technical replicates for the early isoprenoid biosynthetic transcripts in different organs. For *TPS* transcript analysis in trichomes, three biological and three technical replicates were performed. Gene expression was analyzed using qBase+. Statistical analysis was performed by ANOVA on log-transcript abundance, with Bonferroni correction.

TPS gene prediction and phylogeny

`Finola' genome and transcriptome assemblies [24] were downloaded from the cannabis genome browser (http://genome.ccbr.utoronto.ca/cgi-bin/hgGateway). These assemblies were used as the subject of a tBLASTn search using 71 *TPS* genes (S3 Table) downloaded from GenBank and Phytozome. Gene and splice site prediction was performed on scaffolds containing regions with similarity to *TPS* sequences using the Exonerate gene prediction algorithm [34]. A preliminary Purple Kush genome assembly based on PacBio (www.pacb.com) sequencing data was also used. Predicted genes were manually curated against earlier Purple Kush sequence data, and examined to establish open reading frames, start codons, and stop codons. A maximum likelihood phylogeny was built using phylogeny.fr [35]. The alignment used for input was built using the MUSCLE algorithm with all translated amino acid sequences from the predicted *TPS* gene models from cannabis and the 71 published *TPS* sequences listed above. Alignments were curated using the Gblocks algorithm, and tree construction was performed using PhyML 3.0 with 100 bootstrap replicates.

Results

Terpene profiles of cannabis inflorescences

We used the *C. sativa* oilseed hemp variety `Finola' to investigate terpene profiles of pistillate flowers. `Finola' was chosen because reference draft genome and transcriptome assemblies were published [24]. Pistillate flowers, which have the highest density of glandular trichomes relative to other parts of the plant (Fig 1), were sampled to cover early to mid-stage inflorescences between three and eight weeks post onset of flowering, where onset of flowering is defined as the first appearance of pistils. Independent of the stage of inflorescence, the most abundant monoterpenes were myrcene, (+)-α-pinene, (-)-limonene, (+)-β-pinene, terpinolene, and (*E*)-β-ocimene (Table 1). The most abundant sesquiterpenes were β-caryophyllene, α-humulene, bergamotene, and farnesene. Terpene profiles showed considerable variations

Table 1. Relative composition of terpene profiles in *C. sativa* 'Finola' pistillate flowers. Twenty two individual plants were sampled. Contribution of individual terpenes is expressed as a proportion of the total terpenes within a given class (i.e., monoterpenes or sesquiterpenes).

Metabolite	Percent Proportion (mean ± st. dev)	Terpene class
(+)-α-Pinene	23 ± 17	Monoterpene
(+)-β-Pinene	8.6 ± 4.6	Monoterpene
Myrcene	27 ± 21	Monoterpene
(-)-Limonene	12 ± 10	Monoterpene
(*E*)-β-Ocimene	10 ± 6.5	Monoterpene
Terpinolene	18 ± 14	Monoterpene
β-Caryophyllene	46 ± 13	Sesquiterpene
Bergamotene	3.6 ± 3.0	Sesquiterpene
Farnesene	4.4 ± 3.6	Sesquiterpene
α-Humulene	19 ± 7.6	Sesquiterpene

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between individual plants as indicated with the relatively high standard deviation (Table 1). No trends were observed for individual metabolites as a function of inflorescence development, but total monoterpenes increased compared to sesquiterpenes as inflorescences matured. Mid-stage flowers (~4 weeks post onset of flowering) had a mean monoterpene content of 389 μg g^1 DW (SE = 44, n = 9), and a mean sesquiterpene content of 34 μg g^1 DW (SE = 6.3, n = 9).

Transcriptome mining of early isoprenoid biosynthesis genes

We queried the `Finola' transcriptome for transcripts involved in the early stages of isoprenoid biosynthesis. We combined four transcriptome sets downloaded from the Cannabis Genome Browser (http://genome.ccbr.utoronto.ca/cgi-bin/hgGateway), including transcripts from developing seeds, mature pistillate flowers, stamenate (male) flowers, and whole seedlings. The tBLASTn algorithm was used to search translated `Finola' nucleotide sequences, using amino acid sequences from *Vitis vinifera* and *Arabidopsis thaliana*, and an e-value cut-off of 1^{-10}.

At least one full-length or nearly full-length (>95%) transcript was found for each of the core genes in the MEP and MEV pathways, and linear isoprenoid diphosphate prenyltransferases (Fig 2B). The genes included in the analysis of the MEP pathway were 1-deoxy-D-xylulose 6-phosphate (DOXP) synthase (DXS), DOXP reductoisomerase (DXR), 2-C-methyl-D-erythritol cytidyltransferase (MCT), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-hydroxy-3-methyl-but-2-enyl diphosphate (HMB-PP) synthase (HDS), and HMB-PP reductase (HDR). Two versions of DXS, CsDXS1 and CsDXS2, were found, which are 62.8% identical at the amino acid level. In a phylogeny, CsDXS1 clusters with members of the DXS subfamily DXS-I of other plant species, and CsDXS2 clusters with members of the DXS-II subfamily (S1 Fig).

The genes included in the MEV pathway analysis were 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (HMGS), HMG-CoA reductase (HMGR), mevalonate kinase (MK), phospho-mevalonate kinase (PMK), mevalonate-5-phosphate decarboxylase (MPDC), and IPP isomerase (IDI). At least one transcript was found corresponding to each enzyme. Two transcripts were found for HMGR, HMGR1 and HMGR2, which are 72.7% identical at the amino acid level.

As candidate prenyltransferases, we found transcripts of a heterodimeric GPPS system similar to that characterized in hop [17], with a GPPS large subunit (GPPS.lsu) and a GPPS small subunit (GPPS.ssu). Two transcripts were identified corresponding to FPPS, 80.3% identical to one another at the amino acid level.

RT-qPCR expression analysis of isoprenoid biosynthetic transcripts

To assess gene expression of isoprenoid biosynthesis across different parts of the cannabis plant, we used qRT-PCR to examine the transcript abundance of prenyltransferases and key genes in the MEP and MEV pathways. We selected genes for three steps in the MEP pathway, *DXS*, *DXR*, and *HDR*, as well as two MEV pathway genes, *HMGR* and *IDI*. We also included the two *FPPS* genes. In heterodimeric GPPS, the rate of GPP biosynthesis is governed by ratios of small to large subunits, with higher ratios of small to large subunits leading to higher GPP formation [17]. We therefore measured transcript levels of the *GPPS.ssu* gene. Transcript levels were determined in `Finola' leaves, stems, roots, staminate flowers, and glandular trichomes from pistillate flowers. Pistillate flowers were harvested between 10±12 weeks post germination.

CsDXS1 was expressed in all samples, with no significant differences between different parts of the plant (Fig 2B). *CsDXS2* was also expressed in all samples. Levels of *CsDXS1* and *CsDXS2* transcripts were not significantly different except in roots, where average *CsDXS2* levels were 14-fold more abundant than *CsDXS1*. *HMGR1* and *HMGR2* were both expressed in every sample. Their transcript abundances were not significantly different, except in leaves and roots where *HMGR2* was significantly more highly expressed. Abundances of *FPPS* transcripts in roots and staminate flowers were significantly different between the two *FPPS* genes. The MEP pathway genes *DXR* and *HDR* were significantly more highly expressed in trichomes and leaves. Cannabis leaves bear glandular trichomes, but much less densely than flowers. Genes in the MEP pathway were also more highly expressed in trichomes and leaves than genes of the MEV pathway. Transcripts of *GPPS.ssu* were very highly abundant (>25 fold higher) in trichomes compared to other tissues.

Members of the cannabis *TPS* gene family

In the `Finola' (FN) trichome transcriptome we identified nine full-length or nearly full-length (predicted >95% of amino acid length) and six partial putative *TPS* genes (*CsTPS FN*). A maximum likelihood phylogeny of the nine full-length CsTPSFN predicted protein sequences and representative TPS from other plant species placed the CsTPSFN most closely with each other and with TPS from hop (HlSTS1 and HlSTS2) indicating a recent expansion of *TPS* genes in the *Cannabaceae* (Fig 3). Five of the nine CsTPSFN (CsTPS1FN, CsTPS2FN, CsTPS3FN, CsTPS5FN, CsTPS6FN) clustered with members of the TPS-b subfamily, and the remaining four (CsTPS4FN, CsTPS7FN, CsTPS8FN, CsTPS9FN) clustered with the TPS-a subfamily. Two of the CsTPSFN TPS-b genes, CsTPS1FN and CsTPS2FN, encode predicted proteins that were 98.7% and 96.8% identical to CsTPS1 and CsTPS2 previously reported [36] and identified there as (-)-limonene synthase (CsTPS1) and (+)-α-pinene synthase (CsTPS2) from the *C. sativa* strain `Skunk'.

Functional characterization of *CsTPSFN* TPS-b subfamily members

CsTPSFN were cloned as cDNAs from `Finola' pistillate flowers or synthesized for heterologous expression and identification of product profiles of the encoded enzymes. We cloned four TPS-b family members, *CsTPS1FN*, *CsTPS2FN*, *CsTPS5FN*, and *CsTPS6FN*, from cDNA. *CsTPS3FN* could not be cloned from cDNA and was obtained as a synthetic cDNA. Three TPS-b sequences from the Purple Kush (PK) trichome transcriptome, *CsTPS13PK*, *CsTPS30PK*, and *CsTPS33PK*, were also synthesized for comparison. These five CsTPS from `Finola' and three from Purple Kush were expressed as recombinant proteins and then tested for activity with GPP and FPP and products identified by GC-MS analysis (Fig 4, Table 2, S2 and S3 Figs).

The major product of CsTPS1FN was (-)-limonene, with minor products of (+)-α-pinene, camphene, (+)-β-pinene, and myrcene (Fig 4). CsTPS2FN produced mostly (+)-α-pinene, with minor amounts of (+)-β-pinene, myrcene, (-)-limonene, β-phellandrene and a monoterpene

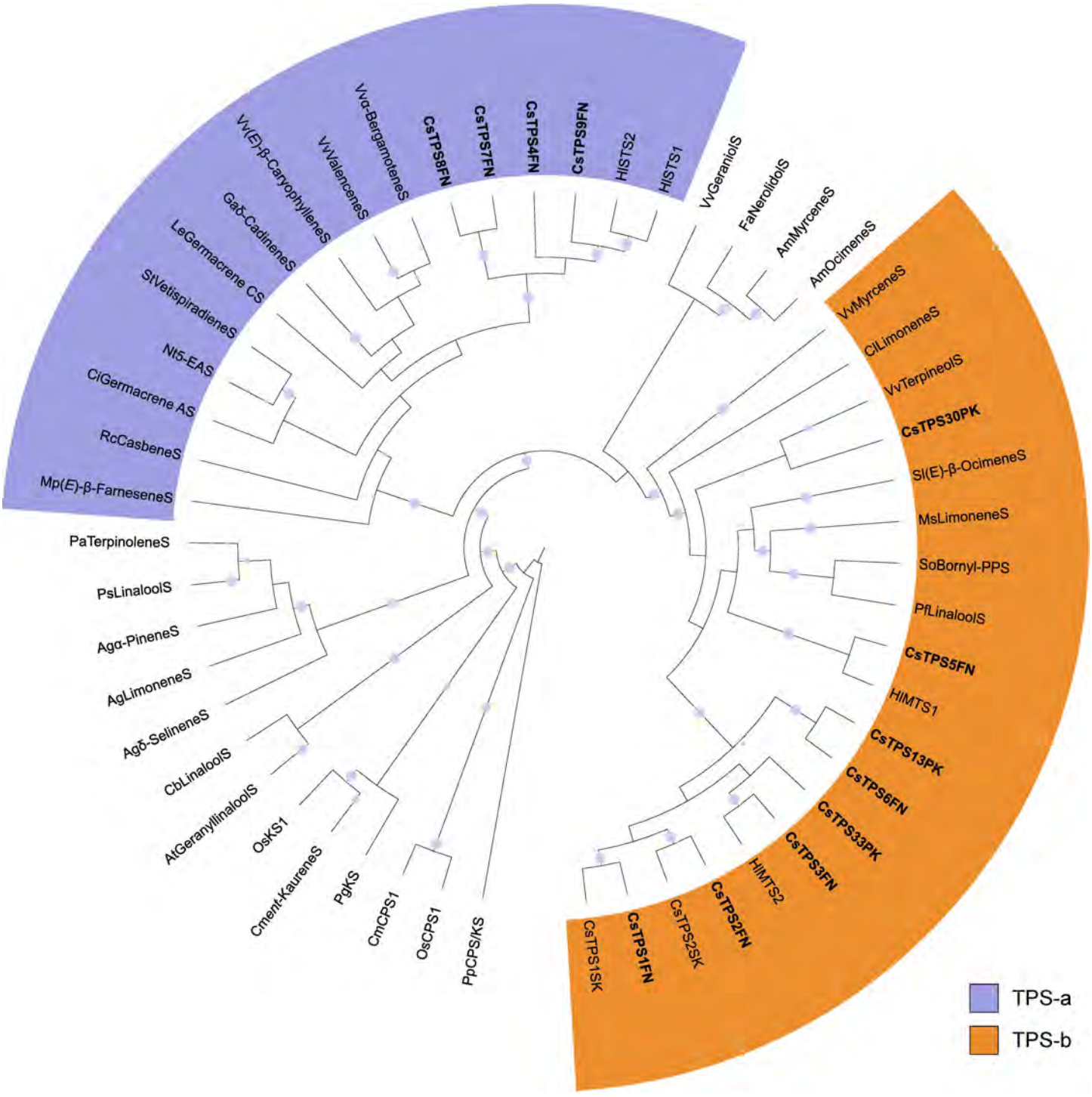

Fig 3. Maximum likelihood phylogeny of CsTPS. Within the TPS-a and TPS-b subfamilies, TPS from the *Cannabaceae*, including cannabis and hops, are more closely related to one another than to TPS from other angiosperms. Cannabis TPS are in bold. The cannabis strain or variety of origin is indicated by two letters following the TPS#: FN: `Finola', SK: `Skunk', PK: Purple Kush. Branches with bootstrap values >80% (100 repetitions) are indicated with a grey dot. TPS of other species included are from Pp: *Physcomitrella patens*, Os: *Oryza sativa*, Cm: *Cucurbita maxima*, At: *Arabidopsis thaliana*, Cb: *Clarkia breweri*, Ag: *Abies grandis*, Pa: *Picea abies*, Fa: *Fragaria ananassa*, Am: *Antirrhinum majus*, Mp: *Mentha x piperita*, Rc: *Ricinus communis*, Ci: *Cichorium intybus*, Sl: *Solanum lycopersicum*, Nt: *Nicotiana tabacum*, Le: *Lycopersicum esculentum*, Ga: *Gossypium arboreum*, St: *Solanum tuberosum*, Vv: *Vitis vinifera*, Hl: *Humulus lupulus*, So: *Salvia officinalis*, Cl: *Citrus limon*, Ms: *Mentha spicata*, Pf: *Perilla frutescens*. `S' suffix = synthase.

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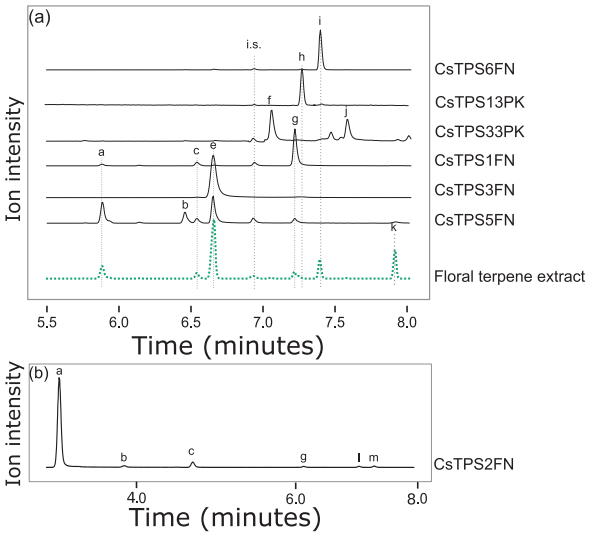

Fig 4. Representative GC-MS traces showing products of *CsTPSFN* TPS-b subfamily members. Black traces show GC-MS total ion chromatogram from CsTPS assays with GPP. Green trace, dotted line, is a representative terpene profile from a `Finola' inflorescence. (a) shows representative chromatograms from six TPS and a `Finola' floral extract run on an HP-5 GC column. (b) shows the representative chromatogram from CsTPS2FN run on a DB-Wax GC column. Peaks: a) α-pinene, b) camphene, c) sabinene, d) β-pinene, e) myrcene, f) α-terpinene, g) limonene, h) (*Z*)- β-ocimene, i) (*E*)-β-ocimene, j) γ-terpinene, k) terpinolene, l) β -phellandrene, m) isoterpinolene. i.s. = internal standard

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tentatively identified as isoterpinolene (Fig 4). CsTPS3FN produced myrcene as a single detectable product when incubated with GPP (Fig 4). CsTPS30PK also produced only myrcene when tested with GPP (S2 Fig). These two single-product myrcene synthases share only 54.5% amino acid identity. CsTPS5FN also produced myrcene as its most abundant monoterpene product (37%) (Fig 4), but unlike CsTPS3FN and CsTPS30PK, CsTPS5FN produced four additional

Table 2. Functionally characterized CsTPS enzymes.

Functional gene ID	Nearest 'PK' *TPS* gene model	Major products	Strain of origin	Activity on GPP	Activity on FPP
CsTPS1FN	CsTPS1PK	(-)-limonene	Finola	+++	np
CsTPS1SK[‡]	CsTPS1PK	(-)-limonene	Skunk	nd	nd
CsTPS2FN	CsTPS2PK	(+)-α-pinene	Finola	+++	np
CsTPS2SK[‡]	CsTPS2PK	(+)-α-pinene	Skunk	nd	nd
CsTPS3FN	CsTPS3PK	β-myrcene	Finola	+++	np
CsTPS4FN	CsTPS9PK	alloaromadendrene	Finola	+	+++
CsTPS5FN	CsTPS5PK	β-myrcene, (-)-α-pinene	Finola	+++	+
CsTPS30PK	CsTPS30PK	β-myrcene	Purple Kush	+++	+
CsTPS6FN	CsTPS6PK	(*E*)-β-ocimene	Finola	+++	np
CsTPS7FN	CsTPS7PK	δ-selinene*	Finola	+	+++
CsTPS8FN	CsTPS8PK	γ-eudesmol*, valencene	Finola	+	+++
CsTPS9FN	CsTPS9PK	β-caryophyllene, α-humulene	Finola	+	+++
CsTPS13PK	CsTPS13PK	(Z)-β-ocimene	Purple Kush	+++	+
CsTPS33PK	CsTPS33PK	α-terpinene, γ-terpinene	Purple Kush	+++	np

[‡]Published in Gunnewich et al., 2008

*Product not compared to authentic standard.

np, no product detected. nd, no data available.

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monoterpenes (-)-α-pinene (23%), (-)-limonene (17%), sabinene (15%), and (-)-β-pinene (8%). The same product profile was identified when CsTPS5FN was transiently expressed in *N. benthamiana* (S4 Fig). CsTPS5FN was somewhat unusual among TPS-b members in lacking any obvious N-terminal plastidial targeting sequence. CsTPS5FN also produced minor amounts of farnesene when incubated with FPP, making it the only member of the TPS-b subfamily to produce detectable sesquiterpenes. CsTPS6FN produced 97% (*E*)-β-ocimene with GPP, and the remaining 3% of product was (*Z*)-β-ocimene. A TPS sequence found in Purple Kush, CsTPS13PK, shares 95.5% amino acid sequence identity with CsTPS6FN. CsTPS13PK produces 94% (*Z*)-β-ocimene. A third TPS from Purple Kush, CsTPS33PK, produced two different monoterpenes, α-terpinene (61%) and γ-terpinene (39%) (Fig 4).

Functional characterization of *CsTPSFN* TPS-a subfamily members

Four TPS-a family members cloned as cDNAs from `Finola', *CsTPS4FN*, *CsTPS7FN*, *CsTPS8FN*, and *CsTPS9FN*, were expressed as recombinant proteins, proteins tested with GPP and FPP and products identified by GC-MS (Fig 5A, Table 2). CsTPS4FN produced mostly alloaromadendrene (52.3% of total products) with FPP (Fig 5C). The remaining products are a mixture of five sesquiterpene olefins and two alcohols, including valencene, α-humulene, and a product tentatively identified as palustrol. CsTPS4FN was also active with GPP, producing minor amounts of myrcene (S5 Fig). CsTPS7FN produced 21 sesquiterpene olefins and two sesquiterpene alcohols. Of these, products tentatively identified as δ-selinene and selina-6-en-

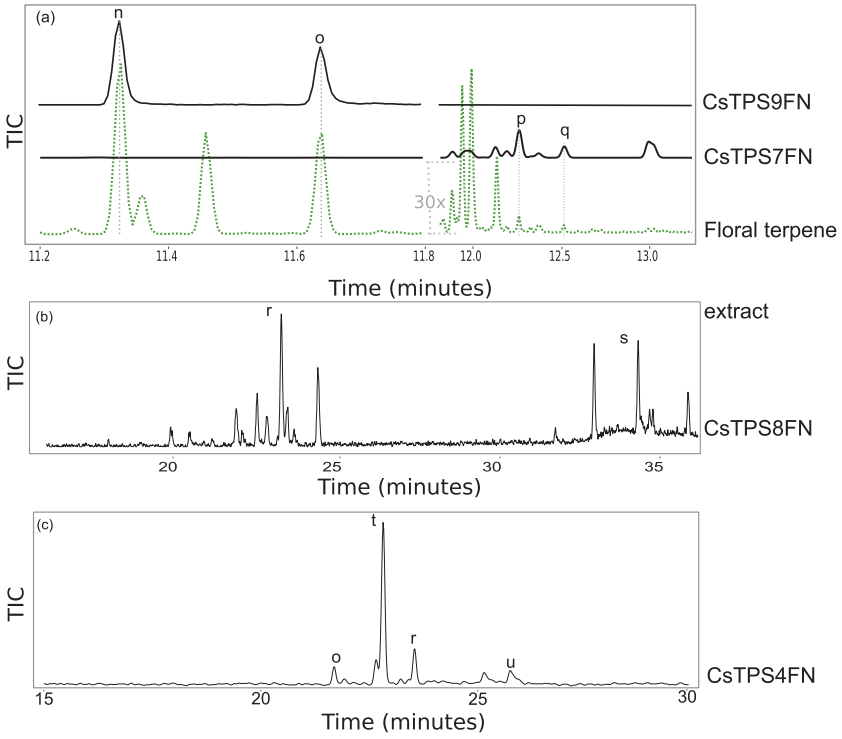

Fig 5. Representative GC-MS traces showing products of *CsTPSFN* TPS-a subfamily members. Black traces show GC-MS total ion chromatogram (TIC) from CsTPS assays with FPP. Green trace, dotted line, in (a) is representative terpene profiles from `Finola' inflorescences. The right-hand region of the `Finola' terpene profile has been amplified 30-fold to facilitate comparison with the products of CsTPS7FN. (b) shows the trace of CsTPS8FN after cold injection (40ÊCinlet) onto a DB-wax column. (c) shows the trace for CsTPS4FN on an HP-5 column. Peaks: n) β-caryophyllene, o) α-humulene, p) δ-selinene, q) selina-6-en-4-ol, r) valencene, s) γ-eudesmol, t) alloaromadendrene, u) palustrol.

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4-ol make up 20.5% and 13.9% of the product profile, respectively. Very few of `Finola' individuals tested contained minor amounts of the products of CsTPS7FN (Fig 5A). The remaining minor products each make up <10% of total sesquiterpene products. When incubated with GPP, CsTPS7FN produced myrcene and limonene (S5 Fig). The most abundant product of CsTPS8FN was initially identified as β-elemol (S6 Fig), which is often an artifact of heat-induced rearrangement. Using a lower injection temperature of 40ÊC,the β-elemol product was no longer detected and was replaced by peaks corresponding to 11 sesquiterpene olefins and three sesquiterpene alcohols. Of these, two of the major products were identified as γ-eudesmol (19.8%) and valencene (19.6%) (Fig 5B). When CsTPS8FN was incubated with GPP limonene and myrcene were detected (S5 Fig).

CsTPS9FN produced β-caryophyllene and α-humulene from FPP (Fig 5A). These two terpenes are always the most abundant sesquiterpenes in cannabis resin terpene profiles. The CsTPS9FN enzyme produces these two sesquiterpenes in a ratio of approximately 2.5 to 1, which is similar to the ratio of 2.4 +/- 0.2 to 1 observed in `Finola' terpene profiles.

CsTPS transcripts are highly abundant in pistillate inflorescences

To determine to what extent the Cs*TPS* genes described above contribute to the trichome terpene profile, we performed RT-qPCR on transcripts of five *CsTPS* in glandular trichomes isolated from pistillate flowers. Transcript levels of *CsTPS1FN*, *CsTPS2FN*, *CsTPS3FN*, *CsTPS6FN*, and *CsTPS9FN* were examined in trichomes (S7 Fig) isolated from eight `Finola' individuals between two and four weeks post onset of flowering. These five *CsTPS* were chosen because they have a single or at most two products, thus it was deemed more likely to be possible to attempt correlating metabolite abundance with transcript abundances than would be possible with the multiproduct *CsTPS*.

Of the eight individual plants, seven showed typical inflorescence terpene metabolite profiles. Surprisingly, one individual had no detectable inflorescence monoterpenes except for traces of (*E*)-β-ocimene, although it did contain cannabinoids and sesquiterpenes in floral trichomes (S8 Fig). In Fig 6A, metabolite levels of the target compounds are expressed as a proportion of the total mono- or sesquiterpenes in each floral terpene extract. *CsTPS2FN* was the most abundant of the six different *TPS* transcripts measured, and its major product, (+)-α-pinene, was also the most abundant monoterpene on average in the eight plants examined. Similarly, (*E*)-β-ocimene and the (*E*)-β-ocimene synthase CsTPS6FN were the least abundant of all metabolites and transcripts measured, respectively. However, within transcript/metabolite pairs, only the correlation between (-)-limonene and *CsTPS1FN* transcript level was significant (Fig 6A). Correlation between metabolite level and transcript abundance was not significant for any of the other metabolite/transcript pairs.

In addition, we measured the transcript abundance of the multiproduct monoterpene synthase *CsTPS5FN*, to assess if its expression may contribute to terpene profiles in the resin. *CsTPS5FN* transcripts were highly abundant in some individuals, comparable to the highest transcript levels of any other *CsTPS* tested (Fig 6B). Transcript levels of this gene did not explain any of the lack of correlations between the five terpene-metabolite pairs tested above. Additionally, plant X, which had no detectable monoterpenes, had moderate levels of *CsTPS5FN* transcript. It appears that *CsTPS5FN*, while highly expressed, does not contribute to terpene accumulation in `Finola'.

Discussion

The resin of *C. sativa* is rich in mono- and sesquiterpenes, which are of interest for their putative contributions to cannabis pharmacology [6]. Most studies of terpenes in cannabis have

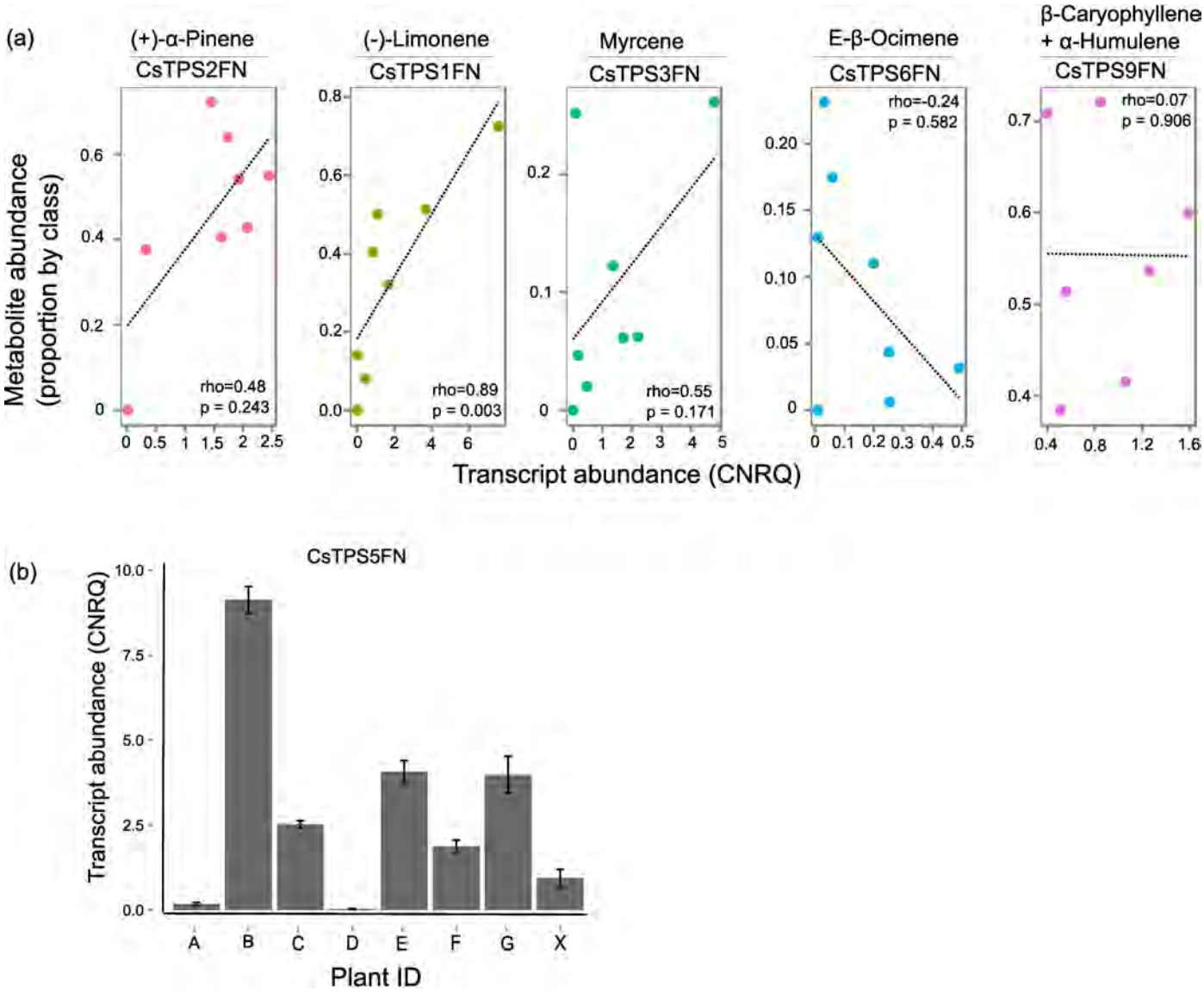

Fig 6. Correlation analysis of metabolite abundance in inflorescence and transcript abundance for five CsTPS in isolated trichomes. (a) Data are shown for five CsTPS/metabolite pairs each in eight `Finola' individuals. Metabolites given with their relative abundance were those that match the product of the corresponding CsTPS. Plant `X' was not included in the left-most panel. Metabolite abundances are expressed as a proportion of the total mono- or sesquiterpenes for each individual. Transcript abundances are calibrated normalized values compared to two reference genes. rho = Spearman rank correlation between transcript and metabolite abundances, p value indicates significance. (b) Transcript abundance of CsTPS5FN in eight `Finola' individuals.

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focused on phytochemical composition for forensics and breeding, while less research has gone into the molecular biology of terpene formation in cannabis. Knowledge of the genomics and gene functions of terpene biosynthesis may facilitate genetic improvement of cannabis for desirable terpene profiles. Using the hemp strain `Finola' and its genome and transcriptome resources [24], we identified early isoprenoid pathway genes as well as specific *CsTPS* genes and their enzymes involved in the biosynthesis of nearly all of the different monoterpenes identified in extracts of the cannabis inflorescences, which are densely covered with terpene and cannabinoid accumulating glandular trichomes (Fig 1). One exception is terpinolene, for which a *CsTPS* has not yet been identified. The terpene profiles of cannabis can be explained

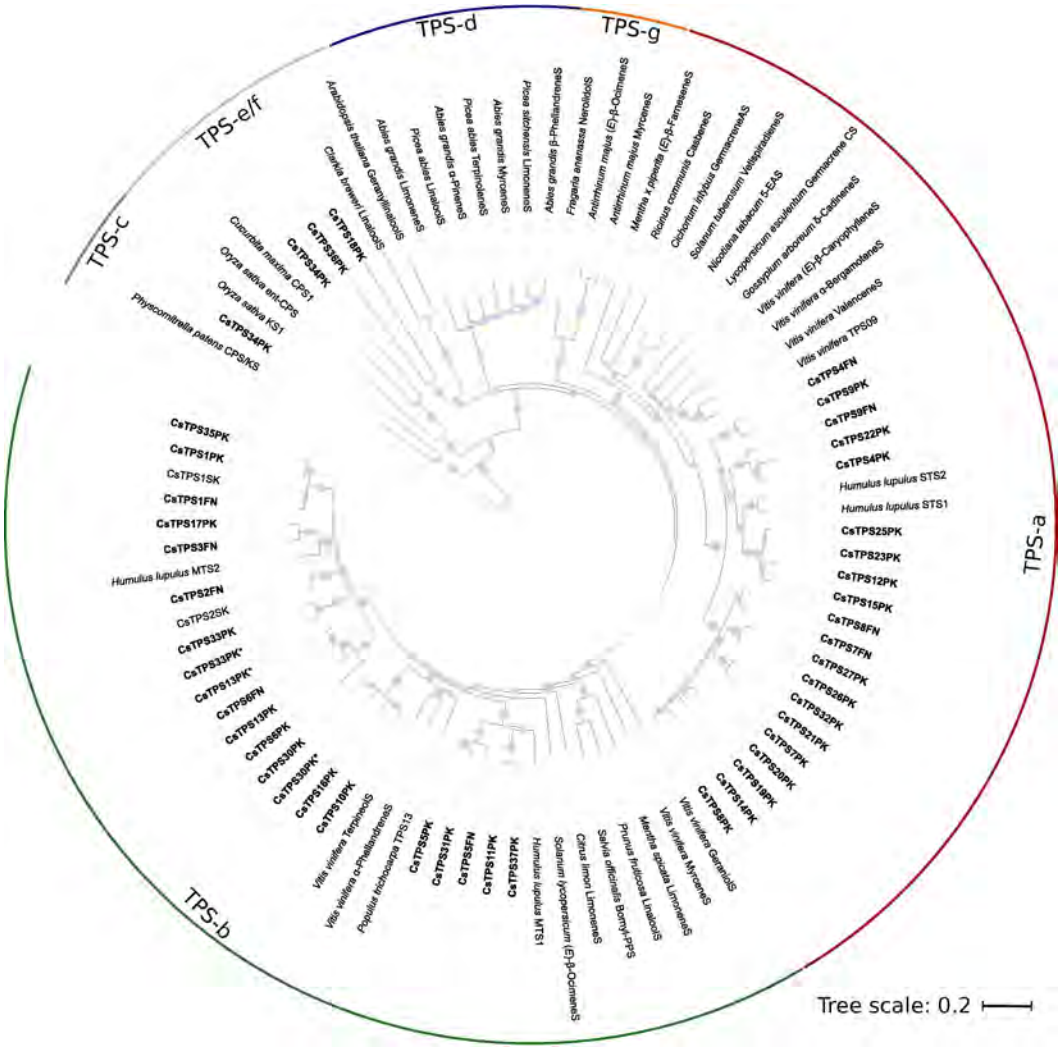

Fig 7. Maximum likelihood phylogeny for 33 TPS translated from gene models identified in the *Cannabis sativa* Purple Kush genomic sequences. 41 published TPS sequences from other organisms were included for comparison. Names of cannabis genes identified in this study are in bold. Gene names from Purple Kush followed by an asterisk (*) represent biochemically characterized enzymes from Purple Kush transcriptome data. Their nearest homologue in the genome was assigned the same gene ID when the sequences had >95% amino acid identity. Branches with greater than 80% boostrap support are identified with a grey circle.

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by activities of both single-product and multi-product *CsTPS*. Individual `Finola' plants showed substantial variation in their profiles of mono- and sesquiterpenes. `Finola' has few monoterpene alcohols or ethers, such as linalool or geraniol, which are common in some cannabis strains.

It is reasonable to expect that there are additional *CsTPS* not described in this work, such as a *CsTPS* that encodes a terpinolene synthase. Characterization of further TPS may also clarify the poor correlation between TPS and the abundance of their products shown in Fig 6A. A search of a new assembly of the Purple Kush genome, to which we recently had pre-publication access (Dr. Jonathan Page, personal communication), identified a total of 33 complete *CsTPSPK* gene models and additional partial sequences (Fig 7). Purple Kush is a marijuana strain which requires special research licensing to grow. Thus, characterization of this more

comprehensive set of *CsTPSPK* will have to be completed in future work as it requires synthesized genes.

Fig 7 indicates a set of putatively orthologous *CsTPSFN* and *CsTPSPK* genes, which may contribute to overlapping terpene profiles in hemp and marijuana varieties. However, some orthologous genes may have evolved different functions in different strains, and non-orthologous *CsTPS* may contribute to some of the same terpene products in different cannabis strains. For example, α-pinene is a major component of strains reported as Purple Kush [37], but no obvious orthologue of the α-pinene synthase CsTPS2 as identified in the `Finola' and `Skunk' strains was found in the Purple Kush genome (Fig 7). Another example is the set of apparently non-orthologous single-product myrcene synthases, CsTPS3FN and CsTPS30PK identified in `Finola' and Purple Kush, which only share 52.5% amino acid identity but produce the same monoterpene. Also, not all *CsTPS* are expected to contribute to terpene accumulation in the resin of cannabis inflorescences and some may function in a different context of the plant biology. For example, *CsTPS5FN* is expressed in inflorescences and the recombinant enzyme produces a mixture of monoterpenes, but does not contribute substantially to the terpene profile of the resin. This gene appears most closely related to *MTS1* from hops (Fig 3) where the encoded protein was inactive *in vitro* [38].

Cannabis inflorescences are densely covered with glandular trichomes, which are specialized to produce and accumulate terpenes [11]. Transcripts of several *CsTPS* genes (Fig 6A) are abundant in trichomes isolated from mid-stage `Finola' inflorescences. Transcripts associated with early isoprenoid biosynthesis and especially the MEP pathway, which feeds into both monoterpene and cannabionoid biosynthesis, were also abundant in trichomes (Fig 2B). Sesquiterpenes have been reported to be most abundant in early floral stages [39], and thus MEV pathway transcripts may be more abundant at earlier stages of flower development. Different DXS and HMGR genes were differentially expressed in roots relative to other parts of the plant. Terpenes in the roots, if present in cannabis, may contribute to defense as reported in other plant species [10]. In plants, *DXS* genes generally fall into two clades, of which DXS I members are generally involved in primary metabolism, and DXS II members are often induced in defense responses [40, 41, 42]. Abundance of cannabis *DXS2* transcripts, which clusters with the DXS II subfamily (S1 Fig), suggests defense related terpenoids in cannabis roots and warrants future work on the cannabis root metabolome. We also observed high *FPPS* transcript abundance in stamenate flowers and roots, resembling a previous finding that Arabidopsis FPS1 was primarily expressed in flowers and roots compared to AtFPS2 [43].

Domestication and selective breeding can result in changes in terpene profiles and abundance. For example, domestication can lead to a decrease in the quantity or variability of terpenes [44, 45, 46]. Cannabis, especially marijuana, has been domesticated for thousands of years for increased resin volume and potency [2, 47] and as a result profiles and ecological roles of terpenes in ancestral (i.e., undomesticated) cannabis are unknown. The present study highlights the large number of CsTPS genes and the diverse products of the encoded TPS enzyme activities, which contribute to the complex terpene profiles of cannabis. The knowledge of multigene nature of the CsTPS family and the often multiple products of the encoded enzymes will be critical when selecting or breeding, or improving plants by genome editing, for particular terpene profiles for standardized cannabis varieties. While cannabinoid-free individuals have occasionally been reported [48], we are not aware of any reports in the literature of terpene-free cannabis. In this study, we observed a single monoterpene-free individual, which however still contained cannabinoids and sesquiterpenes. This observation implies that biosynthesis of the different classes of terpenoid metabolites are independently regulated. The fact that terpenes have persisted throughout domestication as a substantial and diverse component of cannabis resin highlights their significance for human preferences.

GenBank accessions

GenBank accession numbers for the terpene synthases described in this paper are CsTPS1FN: KY014557, CsTPS2FN: KY014565, CsTPS3FN: KY014561, CsTPS4FN: KY014564, CsTPS5FN: KY014560, CsTPS6FN: KY014563, CsTPS7FN: KY014554, CsTPS8FN: KY014556, CsTPS9FN: KY014555, CsTPS11FN: KY014562, CsTPS12PK: KY014559, CsTPS13PK: KY014558. Accession numbers for genes in the MEP pathway are CsDXS1: KY014576, CsDXS2: KY014577, CsDXR: KY014568, CsMCT: KY014578, CsCMK: KY014575, CsHDS: KY014570, CsHDR: KY014579. Accession numbers for genes in the MEV pathway are CsHMGS: KY014582, CsHMGR1: KY014572, CsHMGR2: KY014553, CsMK: KY014574, CsPMK: KY014581, CsMPDC: KY014566, CsIDI: KY014569. Prenyltransferase accession numbers are CsGPPS. ssu1: KY014567, CsGPPS.ssu2: KY014583, CsFPPS1: KY014571, CsFPPS2: KY014580. Accession numbers for genomic regions containing putative terpene synthases from Purple Kush are CsTPS1PK: KY624372, CsTPS4PK: KY624361, CsTPS5PK: KY624374, CsTPS6PK: KY624363, CsTPS7PK: KY624368, CsTPS8PK: KY624352, CsTPS9PK: KY624366, CsTPS10PK: KY624347, CsTPS11PK: KY624348, CsTPS12PK: KY624349, CsTPS13PK: KY624350, CsTPS14PK: KY624351, CsTPS15PK: KY624353, CsTPS16PK: KY624354, CsTPS17PK: KY624355, CsTPS18PK: KY624356, CsTPS19PK: KY624357, CsTPS20PK: KY624358, CsTPS21PK: KY624360, CsTPS22PK: KY624360, CsTPS23PK: KY624362, CsTPS24PK and CsTPS25PK: KY624364, CsTPS26PK and CsTPS27PK: KY624365, CsTPS30PK: KY624367, CsTPS31PK: KY624369, CsTPS32PK: KY624370, CsTPS33PK: KY624371, CsTPS34PK: KY624373, CsTPS35PK: KY624375.

Supporting information

S1 Table. Primers used to clone *TPS* genes.
(XLSX)

S2 Table. qPCR primers.
(XLSX)

S3 Table. Accession numbers of TPS sequences used in tblastn and to construct phylogeny.
(XLSX)

S1 Fig. DXS Phylogeny. Maximum likelihood phylogeny of DXS enzymes. *Cannabis sativa* genes are in bold. DXS of other species included are from: At: *Arabidopsis thaliana*; Pt: *Populus trichocarpa*; Os: *Oryza sativa*; Cr: *Chlamydomonas reinhardtii*; Mt: *Medicago truncatula*; Pa: *Picea abies*.
(PNG)

S2 Fig. Representative GC-MS traces of myrcene synthase products.
(PNG)

S3 Fig. Mass spectra of TPS products. Labels [a]Peak a[o] through [a]Peak u[o] correspond to peaks labeled in Figs 4 and 5.
(PNG)

S4 Fig. Products of CsTPS5FN expressed in *E. coli and Nicotiana benthamiana*. Black trace represents products of recombinant enzyme expressed in *E. coli*, green trace represents products of recombinant enzyme expressed in *N. benthamiana.* Leaf images (right) show GFP positive expression control.
(PDF)

S5 Fig. Products of CsTPS with alternative substrates. Members of TPS-a with GPP as substrate are on the left-hand side. Members of TPS-b with FPP as substrate are on the right.
(PDF)

S6 Fig. Hot vs. cold injection of CsTPS8FN products. Top panel represents total ion chromatogram (TIC) with the injection port at 250ÊCon a DB-Wax column. The bottom panel represents TIC with the injection port at 40ÊC,using the same program and the same column.
(PDF)

S7 Fig. Isolated glandular trichome heads.
(PDF)

S8 Fig. Terpene chemotypes of `Finola' flowers. Abundance of five metabolites or metabolite pairs is measured relative to floral weight and an internal standard. Error bars indicate the standard deviation of five metabolite samples taken from each individual.
(PDF)

S9 Fig. Amino acid sequence alignment of functionally characterized CsTPS enzymes.
(PDF)

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Investigation: JB JKB.

Project administration: JB JKB.

Resources: JB JEP.

Supervision: JB JEP.

Writing ± original draft: JB JKB.

Writing ± review & editing: JB JEP JKB.

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Evolution of the Cannabinoid and Terpene Content during the Growth of *Cannabis sativa* Plants from Different Chemotypes

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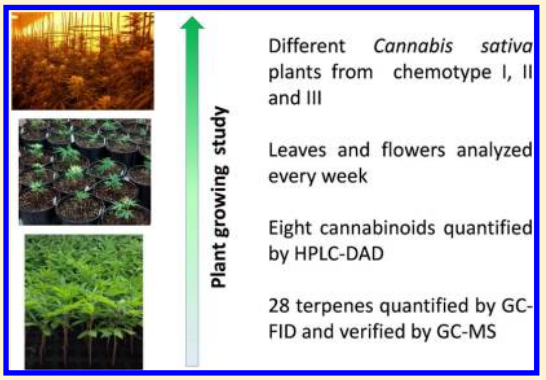
Supporting Information

ABSTRACT: The evolution of major cannabinoids and terpenes during the growth of *Cannabis sativa* plants was studied. In this work, seven different plants were selected: three each from chemotypes I and III and one from chemotype II. Fifty clones of each mother plant were grown indoors under controlled conditions. Every week, three plants from each variety were cut and dried, and the leaves and flowers were analyzed separately. Eight major cannabinoids were analyzed via HPLC-DAD, and 28 terpenes were quantified using GC-FID and verified via GC-MS. The chemotypes of the plants, as defined by the tetrahydrocannabinolic acid/cannabidiolic acid (THCA/CBDA) ratio, were clear from the beginning and stable during growth. The concentrations of the major cannabinoids and terpenes were determined, and different patterns were found among the chemotypes. In particular, the plants from chemotypes II and III needed more time to reach peak production of THCA, CBDA, and monoterpenes. Differences in the cannabigerolic acid development among the different chemotypes and between monoterpene and sesquiterpene evolution patterns were also observed. Plants of different chemotypes were clearly differentiated by their terpene content, and characteristic terpenes of each chemotype were identified.



Cannabis sativa is the most frequently used illicit plant worldwide but is also a highly promising medicinal plant, and its effectiveness for treating various medical conditions has been well documented. For example, it can be used as an appetite-stimulating agent for treating anorexia, cancer, or human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS),[1−3] its antiemetic effects can be beneficial for cancer chemotherapy patients,[4−6] and it provides chronic neuropathic pain relief for cancer, HIV/AIDS, and other types of chronic pain, such as fibromyalgia and rheumatoid arthritis[7−9] and multiple sclerosis.[10−12] Moreover, there are important emerging clinical applications for cannabis, such as in the treatments of several cancers,[13−16] epilepsy,[17,18] Alzheimer's disease,[19,20] Huntington's disease,[21,22] diabetes,[23,24] and Tourette's syndrome.[25,26]

There are at least 554 identified compounds in *C. sativa* L. plants, among them 113 phytocannabinoids[27,28] and 120 terpenes.[29] Cannabinoids are biosynthesized as prenylated aromatic carboxylic acids, and almost no neutral cannabinoid can be found in fresh plants.[30] However, they may convert to their neutral homologues by spontaneous decarboxylation in the presence of light or heat. Moreover, cannabinoids can be oxidized, as in the case of tetrahydrocannabinol (THC), which can be transformed to cannabinol (CBN).[31] The first cannabinoid in the biosynthetic pathway is cannabigerolic acid (CBGA). It is sequentially transformed into tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA), each by a particular synthase (Figure 1).[32] Moreover, monoterpenes and sesquiterpenes are derived from different addition reactions of geranyl ($C_{10}H_{16}$) and farnesyl ($C_{15}H_{24}$) units, respectively.[33]

The two major cannabinoids and those best known for their therapeutic potentials are THC and CBD, i.e., the neutral homologues of THCA and CBDA, respectively. THC is the main psychoactive agent of cannabis and has anti-inflammatory, analgesic, appetite-stimulant, and antiemetic properties.[34] In contrast, CBD can modulate the euphoric effects of THC and has antipsychotic, neuroprotective, anticancer, antidiabetic, and other positive effects, such as the ability to reduce tobacco addiction.[35−39] Moreover, cannabigerol (CBG) and cannabichromene (CBC) seem to be promising compounds for different medical applications. Although they have not been extensively studied, CBG has promising potential for the treatment of glaucoma,[40] inflammatory bowel disease,[41] and prostate carcinoma.[42] CBC has analgesic effects,[43] the potential to stimulate the growth of brain cells,[44] and the ability to normalize gastrointestinal hypermotility.[45]

Terpenes are responsible for the plant's aroma; in addition, they possess specific medical effects and may act synergistically

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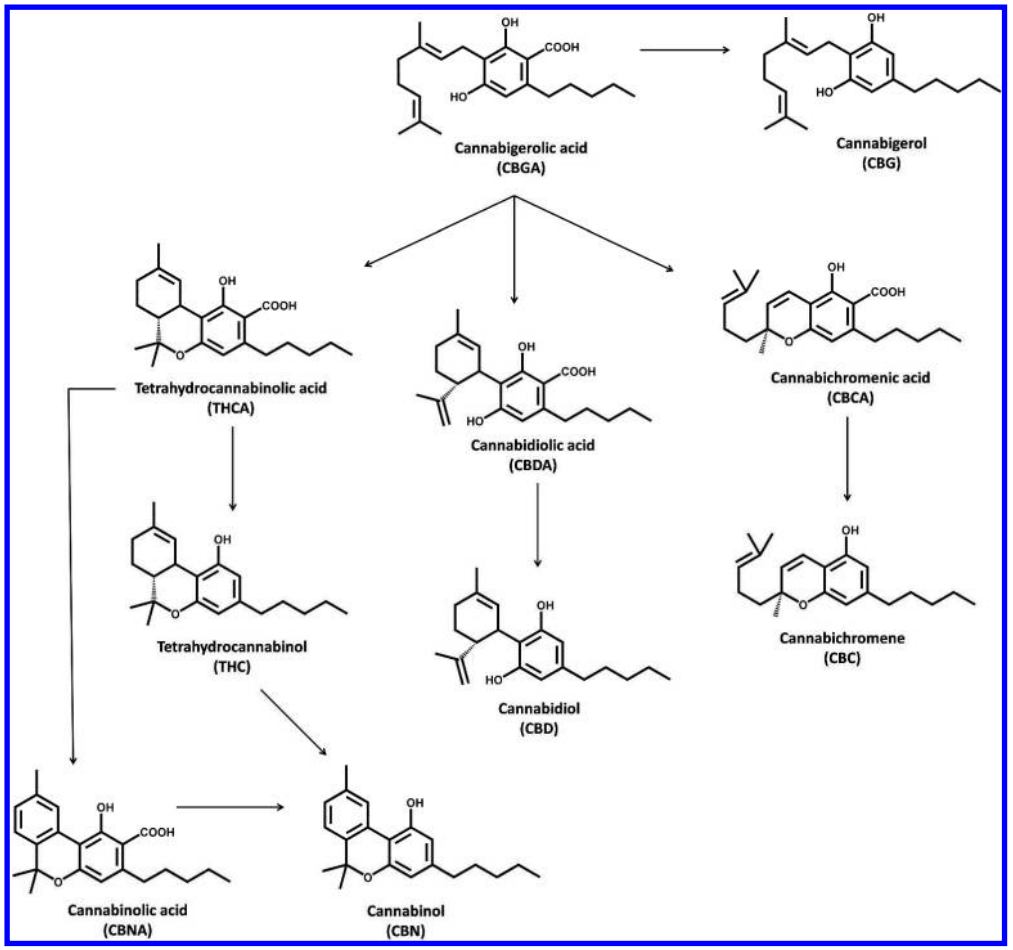

Figure 1. Structures and biosynthetic pathway of the studied cannabinoids.

with cannabinoids. In fact, there are several promising applications based on the combined use of cannabinoids and terpenes, such as new acne therapies utilizing CBD with the monoterpenes limonene, linalool, and pinene; new antiseptic agents with CBG and pinene; treatment of social anxiety disorder using CBD with limonene and linalool; and treatment of sleeping disorders by adding caryophyllene, linalool, and myrcene to 1:1 CBD/THC extracts.[46,47]

Based on the major cannabinoid concentrations, five different chemotypes of cannabis are recognized. Drug-type plants that have a high THCA/CBDA ratio ($\gg$1.0) are classified as chemotype I; plants that exhibit an intermediate ratio (usually 0.5−2.0) are classified as chemotype II; typical fiber-type plants that have a low THCA/CBDA ratio ($\ll$1.0) are classified as chemotype III; chemotype IV plants are fiber-type plants that contain CBGA as the main cannabinoid; and chemotype V plants are also fiber-type plants, but contain almost no cannabinoids.[48]

Genetic analyses have demonstrated that the chemotype is determined by the presence at the B locus of two codominant alleles, B_D and B_T, which are responsible for the CBDA and THCA occurrences in the plant. Thus, plants with B_T/B_T alleles are chemotype I plants, plants with B_D/B_D alleles are of chemotype III, and chemotype II plants have two different alleles, B_D/B_T.[49,50] Nonfunctional alleles, called B_0, can also exist at this locus. These alleles are unable to convert CBGA; hence, these plants are CBGA-predominant (chemotype IV).[51]

The increasing use of cannabis as a medicine and the growing interest in the medicinal effects of nonpsychotropic cannabinoids and terpenes have led to a requirement for the large-scale production of pure compounds and plants with different cannabinoid and terpene content. Thus, CBD-enhanced plants with more than 15% CBD and less than 1% THC have recently been produced.[52] However, to optimize the production for each compound, a better understanding of their development during growth is necessary. Some results regarding this topic have been published, but these applied a forensic perspective to differentiate drug-type plants from nondrug types in the early stages of growth.[48,53] Moreover, the terpene content was not analyzed, and only fiber-type and high-THC plants were studied.

Therefore, the main aim of this work is to study the time evolution of the cannabinoid and terpene content during the entire growth period of the plant, i.e., from the rooting phase until the end of the flowering stage. In this work, the contents of seven cannabis varieties with regard to three different chemotypes (three each from chemotypes I and III and one from chemotype II) are examined.

RESULTS AND DISCUSSION

Figure 2 shows the evolutions of the major cannabinoids, i.e., THCA, CBDA, and CBGA, in the leaves and flowers during the growth of plants from chemotypes I, II, and III. Several conclusions can be drawn from this figure. The chemotypes of the plants, as defined by the THCA/CBDA ratio, were clear from the beginning and stable during growth. This pattern was previously observed in other studies[48,53] and is important for forensic purposes because it does not make sense to wait for plant

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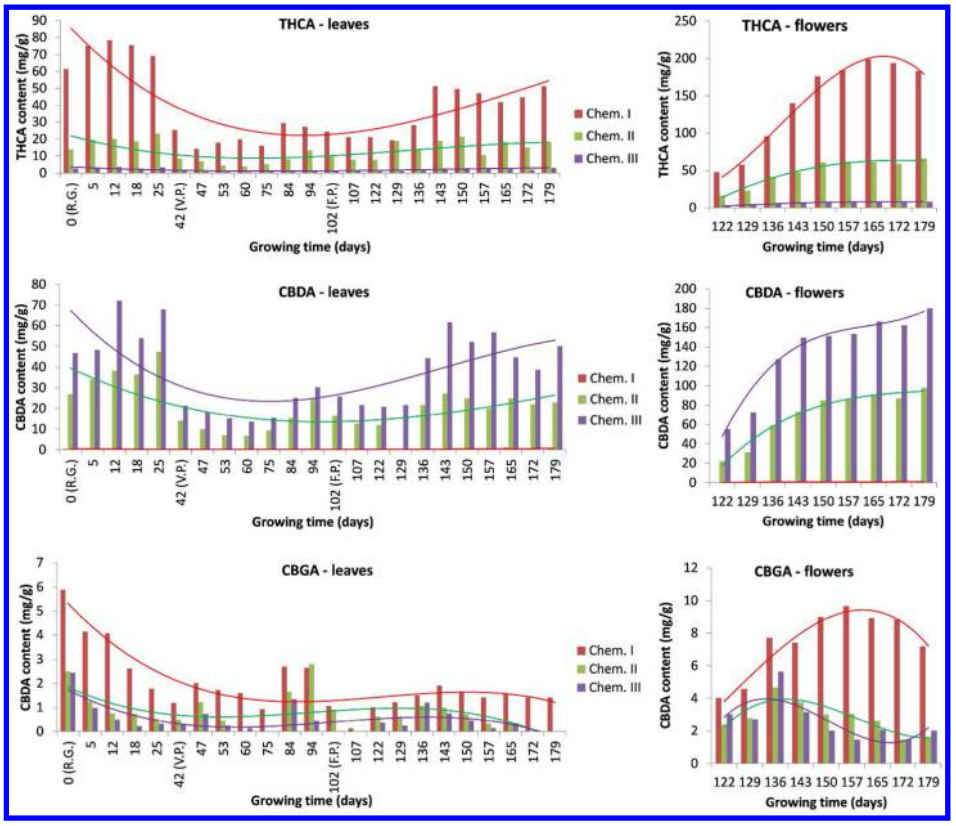

Figure 2. Evolution of the THCA, CBDA, and CBGA content in leaves and flowers during the growth of plants from chemotypes I, II, and III. These values are the averages of all studied plants in each chemotype, with each one measured in triplicate. R.G. refers to the root-growing phase, V.P. to the vegetative phase, and F.P. to the flowering phase.

flowering to identify whether a plant is of a drug type. However, the THCA and CBDA contents in the leaves exhibited the same time evolution for every chemotype. First, the concentrations clearly decreased during the first weeks of the vegetative phase while the plant was growing. Mother plants are well-developed plants that are kept in the vegetative phase. However, as is the case in this study, they are usually kept smaller than plants that are passing to the flowering phase; thus, the cannabinoid content is more concentrated. Therefore, the decrease in the cannabinoid content during plant growth was the expected pattern. In contrast, during the last 2 weeks of the vegetative phase (days 84 and 94), the concentrations of THCA and CBDA increased slightly although the plants did not grow; thus, accumulation of the cannabinoids was observed. Subsequently, a decrease was observed during the first weeks of flowering when the plants were transplanted from 2 L pots to 10 L pots, in which the plants showed further growth. Finally, when trichomes started to develop, an increase was observed not only in the flowers but also in the leaves.

An important observation from Figure 2 is that the maximum concentrations of THCA and CBDA in the flowers were attained at different stages or maturation, depending on the chemotype. For plants of chemotype I, peaks were observed in the ninth week of the flowering phase (day 165), followed by a decrease during the onset of senescence, whereas for chemotype II and III plants, the concentrations continued to increase until conclusion of the study.

Here, CBGA will be discussed. This compound is the first cannabinoid biosynthesized in the plant, and from this compound, THCA, CBDA, and CBCA are synthesized, each by a particular synthase.[32] The CBGA evolution in the leaves was similar to those of THCA and CBDA until the flowering phase started, in which there was a noticeable increase in the THCA and CBDA content but not the CBGA content. As is evident from Figure 2, the concentration of CBGA remained constant in chemotype I plants, whereas it decreased in plants from the other two chemotypes. This difference was more pronounced in the flowers, in which an increase in the CBGA content was observed in chemotype I plants until the onset of senescence, whereas a slight decrease was observed in chemotype II and III plants. This observation was confirmed by statistical data obtained from cross-correlations, in which the correlation coefficients of CBGA with THCA and CBDA during the growth of the plants were found to be 0.789 and −0.114, respectively. In conclusion, the relationship between the CBGA biosynthesis rate and the THCA synthesis rate, controlled by the B_T alleles, was somehow stable during plant growth. In contrast, this relationship was unstable for CBDA synthesis, which was controlled by the B_D alleles.

The minor cannabinoid CBN, derived from THC, was not detected in the plants, indicating that THC did not suffer any measurable degradation. THC, CBD, and CBG, i.e., the neutral compounds of the aforementioned cannabinoids, are not included in Figure 2 because they were found in low concentrations. However, their development was different from those of their acidic cannabinoid homologues. During root growth and the vegetative phase, their content was small (below the limit of quantification), followed by sharp increases in the leaves and flowers during the last 6 weeks of the flowering phase because of decarboxylation of the acidic cannabinoids. A small amount of CBC was found in the plants, and this amount was higher in chemotype I plants. Complete data regarding the

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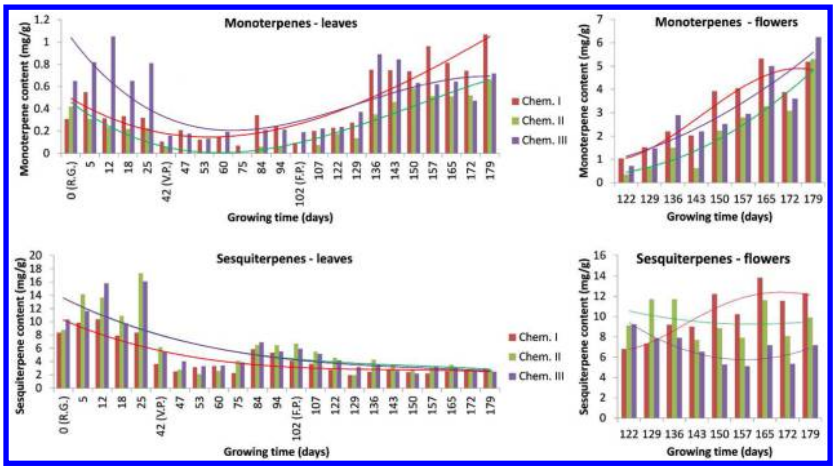

Figure 3. Evolution of total monoterpene and sesquiterpene content in leaves and flowers during the growth of plants from chemotypes I, II, and III. These values are the averages of all studied plants in each chemotype, with each one measured in triplicate. R.G. refers to the root-growing phase, V.P. to the vegetative phase, and F.P. to the flowering phase.

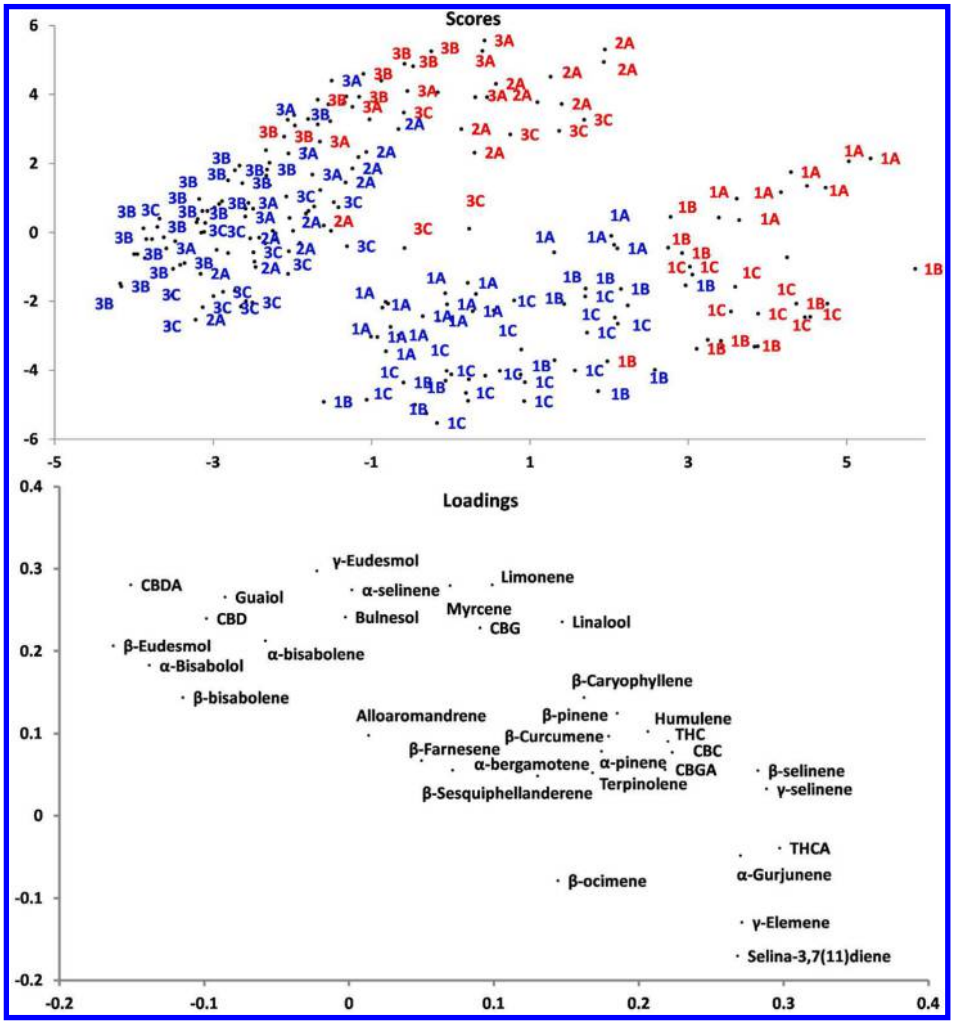

Figure 4. Score and loadings (PC1 vs PC2) obtained via PCA according to the concentrations of all analyzed compounds during the growth of the plants. 1 refers to chemotype I plants, 2 refers to chemotype II plants, and 3 refers to chemotype III plants, whereas A, B, and C denote the different plants of each chemotype. Leaves are colored blue, and flowers are colored red.

evolutions of all studied cannabinoids and terpenes in each plant are available in Tables 1−36 in the Supporting Information.

Figure 3 shows the development of the total monoterpene and sesquiterpene content in the leaves and the flowers of the three different chemotypes. The total monoterpene and sesquiterpene content values were calculated by summing all analyzed terpenes of each kind: eight monoterpenes and 20 sesquiterpenes. The same evolution patterns found for THCA and CBDA in the

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leaves were observed for monoterpenes, i.e., a clear decrease during the first weeks of the vegetative phase, a small increase in the last 2 weeks of the vegetative phase, and a slight decrease during the first weeks of the flowering phase followed by a clear increase. The maximum concentration in the flowers was also chemotype-dependent, and as for THCA and CBDA, this maximum for the total monoterpenes was found in the ninth week of the flowering phase, while for chemotype II and III plants, the concentrations continued to increase until the end of the experiments.

In contrast, sesquiterpenes exhibited a different evolution in both plant matrices. In the leaves, the pattern was similar until the first weeks of the flowering phase, but after that, the content remained stable. In the flowers, the amount of sesquiterpenes did not change significantly during flowering. All terpenes are derived from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The condensation of one DMAPP and two IPP molecules leads to the formation of farnesyl diphosphate (FPP), i.e., the precursor of sesquiterpenes, whereas the condensation of one DMAPP and one IPP molecule leads to the formation of geranyl diphosphate (GPP), i.e., the precursor of monoterpenes. Following the formation of FPP and GPP, sesquiterpenes and monoterpenes are generated by the actions of many specialized terpene synthases (TPSs).[54] However, the expression of these TPSs can differ among the plant tissues and different stages of plant development, thereby resulting in differences in terpene content.[54] Thus, monoterpene synthase expressions were more abundant during this phase, leading to an increase in the monoterpene content during the flowering phase.

To obtain a broader view of the formation of cannabinoids and terpenes, principal component analysis (PCA) and partial least squared regression (PLS) were performed, taking account all of the experimental data (x (224 samples × 36 variables), y (224 samples × 1 time)). Although the PCA model requires more than four PCs to explain the original data structure, up to 60% of the variance is explained by the first three PCs. As observed in Figure 4 in the PC1−PC2 projection, there was a clear distinction between chemotype I plants from the rest (two clusters) and between the leaves and the flowers in each cluster (leaves in blue and flowers in red). The chemotype II plant was closer to the chemotype III plants than the chemotype I plants, most likely because of its higher CBDA content. From the loading projection, the cannabinoids and terpenes of each class of samples were identified as those that are similar to CBDA and THCA. Thus, the higher CBGA and CBC content can be attributed to chemotype I plants. Moreover, terpenes, such as β-eudesmol, γ-eudesmol, guaiol, α-bisabolene, α-bisabolol, or eucalyptol, were much more pronounced in chemotype III plants, whereas γ-selinene, β-selinene, α-gurjunene, γ-elemene, selina-3,7(11)diene, and β-curcumene were characteristic of the chemotype I plants. This chemotype-dependent terpene distribution was also observed in the correlation analysis of the data. As indicated in Table 1, terpenes that were more pronounced in chemotype III plants had higher correlation coefficients with CBDA than with THCA. In contrast, the characteristic terpenes of chemotype I had high correlation coefficients with THCA and negative coefficients with CBDA.

As shown in the 3D score projection (Figure 5), a closer view of the chemotype I plants revealed a fine distinction in the A, B, and C varieties of the plants. Although the leaves and flowers were clearly distinguished by their colors, the class features of each plant were shared in both the leaves and the flowers. Similar results were found for chemotype III plants (data not shown).

Table 1. Correlation Coefficients between the Characteristic Terpenes of Chemotype I and III Plants and THCA and CBDA Obtained via a Cross-Correlation Analysis

chemotype I	THCA	CBDA	chemotype III	THCA	CBDA
γ-selinene	0.921	−0.188	β-eudesmol	−0.160	0.564
β-selinene	0.920	−0.128	γ-eudesmol	0.129	0.517
α-gurgujene	0.858	−0.346	guaiol	−0.109	0.487
γ-elemene	0.790	−0.323	α-bisabolene	−0.151	0.452
selina-3,7(11)-diene	0.704	−0.404	α-bisabolol	−0.293	0.369
β-curcumene	0.702	−0.091	eucalyptol	−0.387	0.365

The development process of the plants was characterized via the PLS analysis, and this characterization was used as a framework to interpret the patterns of the different cannabinoids and terpenes. As was the case in the PCA analysis, the first regression was performed with all of the data, and although the final model with five PCs was able to correlate satisfactorily the main variation pattern with time and to select the most significant variables, the uncertainty of the model was too high to provide a robust interpretation. Therefore, more-restrictive models were built by taking into account only each different type of chemotype. For the chemotype I plants, the final model required five PCs, but the first three explained up to 65% of the variance in x and 88% in y; in the case of chemotype III, the PCs explained 52% of the variance in x and 82% of that in y. The projection of the samples in the PC1−PC2−PC3 space showed the distinction of the three types of plants, as observed before in the PCA model, with a slightly clearer definition of the leaves and the flowers as a consequence of the different maturation processes. The robustness of each PLS model was estimated via a cross-validation procedure and statistical features.[55] Among these features, the regression coefficients of each variable with the growth time were obtained. In Figure 6, the values of the regression coefficients obtained for chemotypes I and III are plotted, including both the significant and nonsignificant coefficients.

From the regression coefficients, it can be observed that neutral cannabinoids exhibited generally higher positive values than acidic cannabinoids, which was in agreement with the increasing concentrations due to decarboxylation as long as the plants were growing. However, these values were clearly influenced by the high level of acidic cannabinoid content in the leaves of the mother plant. In the case of terpenes, clear differences between monoterpenes and sesquiterpenes were found. The monoterpenes exhibited positive coefficients, whereas the sesquiterpenes had mostly negative coefficients, with the clear exception of β-curcumene.

From these results, it is possible to select not only the most acceptable type of plants to produce the target blend of cannabinoids and terpenes but also the growth time needed to fulfill these requirements. In addition, important clues about the biosynthesis rate of CBGA in the different chemotypes and the ratios of this particular cannabinoid with CBDA and THCA were obtained. Finally, it is important to note the relations found with the terpenes because of the synergic effects with cannabinoids and the suitability of this combination for certain therapies.

EXPERIMENTAL SECTION

General Experimental Procedures. The analysis of cannabinoids was performed in an HPLC system that consisted of an Agilent 1100 series chromatograph equipped with a quaternary pump, an

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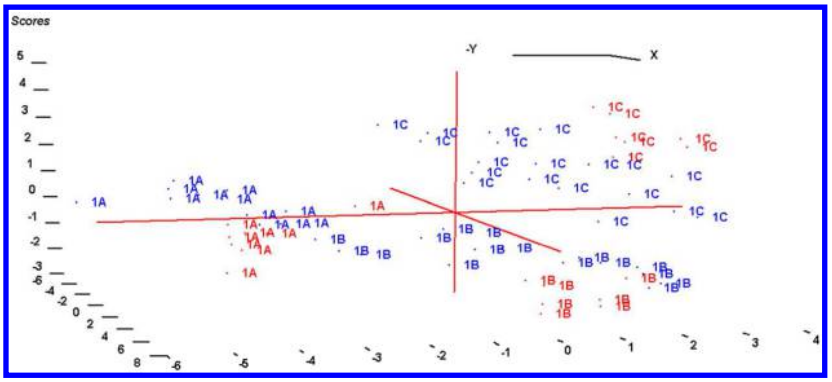

Figure 5. Score loadings (PC1 vs PC2) obtained via PCA according to the concentrations of all analyzed compounds during the growth of chemotype I plants. One refers to chemotype I plants; A, B, and C are the different plants of each chemotype. Leaves are colored blue, and flowers are colored red.

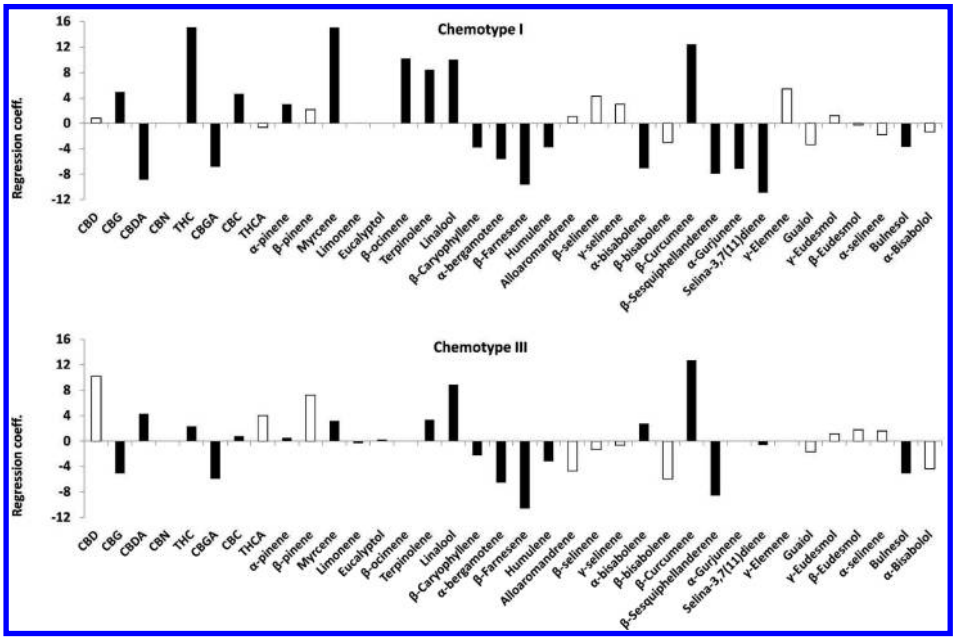

Figure 6. Regression coefficients between the growth time and all studied variables in chemotype I and III plants. Black filled bars are significant variables, and empty bars are nonsignificant ones.

autosampler, and a diode-array spectrophotometer. The analysis was performed according to the Lehmann method with some modifications.[56] Chromatographic separation was achieved using a Nucleosil C_8 column (3 μm, 125 mm × 4 mm i.d.) with a guard column (3 μm depth filter × 4 mm) (Macherey-Nagel, Oensingen, Switzerland) and a binary A/B gradient (solvent A was MeOH, and solvent B was H_2O with 0.1% of HOAc). The gradient program was as follows: the initial conditions were 50% A, which was then increased to 90% A over 20 min, maintained at 90% A over the next 1.5 min, decreased to 50% A over the next 0.5 min, and held at 50% A until 27 min for re-equilibration of the system prior to the next injection. A flow rate of 0.7 mL/min was used, the column was set at 40 °C, and the injection volume was 10 μL. Cannabinoids were quantified at a detection wavelength of 230 nm.

The quantification of cannabinoids was performed with an external calibration, using the average values of three sets of standards containing target compounds at concentrations ranging from 0.5 to 400 μg/mL in MeOH. Low- and high-calibration ranges were used in each set, i.e., 0.5−25 and 10−400 μg/mL, respectively. System fluctuations were corrected with the internal standard phenanthrene at 20 μg/mL, and quality control samples were injected every week along with the samples. The limit of detection and the limit of quantification for all compounds were 0.1 and 0.5 μg/mL, and the correlation coefficients (R^2) were ≥0.9974.

Terpene analysis was performed via GC-MS using an Agilent 6890 series instrument equipped with a 7683 autosampler, a DB5 column (0.25 μm, 30 m × 0.25 mm i.d.) from Agilent Technologies (Santa Clara, CA, USA), and a 5973 single quadrupole mass spectrometer. The transfer line temperature was set to 280 °C, the MS source to 230 °C, and the single quadrupole to 150 °C. The same oven gradient and injection and flow conditions used for the GC-FID were applied, but in this case, helium was used as the carrier gas instead of nitrogen. The analyzed mass range was 50−400 amu. The GC-MS was controlled using the Enhanced Chemstation MSD D.01.00 Build 75 software package (Agilent Technologies), and the NIST 11 library (Standard References Data Program of the National Institute of Standards and Technology, distributed by Agilent Technologies) was used for compound identification. The terpenes α-pinene, β-pinene, myrcene, limonene, eucalyptol, terpinolene, linalool, β-caryophyllene, humulene, and guaiol were identified by comparing their retention times and the obtained mass spectra with reference standards, whereas β-ocimene, α-bergamotene, β-farnesene, alloaromandrene, β-selinene, γ-selinene, α-bisabolene, β-bisabolene, β-curcumene, β-sesquiphellandrene, α-gurjunene, selina-3,7(11)diene, γ-elemene, γ-eudesmol, β-eudesmol, α-selinene, bulnesol, and α-bisabolol were identified using the NIST library.

Finally, an Agilent GC 6890 series equipped with a 7683 autosampler, a flame ionization detector, and a DB5 column (0.25 μm, 30 m × 0.25 mm i.d.) from Agilent Technologies was used for quantitative analysis of terpenes. The analysis was performed according to the Fischedick

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method with minor modifications.[57] The injector temperature was set to 230 °C, the injection volume was 4 μL, and a split ratio of 1:20 was used. A carrier gas (N_2) at a flow rate of 1.2 mL/min was used. The oven temperature program started at 60 °C with a ramp rate of 3 °C/min until 220 °C was reached, and then the temperature was increased to 300 °C with a ramp rate of 40 °C. The temperature was held for 10.67 min at 300 °C, resulting in a total run time of 66 min/sample. The FID detector temperature was set to 250 °C. A H_2 flow of 30 mL/min, synthetic air flow of 400 mL/min, and N_2 make up flow of 25 mL/min were used.

Because of the low variability in the response factors of similar molecular masses,[57] the quantification of all terpenes was performed using the average of three sets of standards containing γ-terpinene at concentrations ranging from 0.5 to 1000 μg/mL in EtOH. Low- and high-calibration ranges were prepared in each set, i.e., 0.5−50 and 25−1000 μg/mL, respectively. System fluctuations were corrected with the internal standard, i.e., 1-octanol, at 100 μg/mL, and quality control samples were injected every week along with the samples. The limit of detection and the limit of quantification for all compounds were 0.1 and 0.5 μg/mL, respectively, and the correlation coefficients (R^2) were ≥0.9998.

Plant Material. Approximately 50 clones of each standardized mother plant (three each from chemotypes I and III and one from chemotype II) were grown indoors under controlled conditions (20−28 °C and 40−70% humidity). Cannabis plants were grown in three cycles. In the beginning, they were cultivated in 25 mm × 25 mm slabs until the roots grew and then were transferred to 2 L pots. During these two steps, the plants were grown under an indoor vegetative light cycle of 18 h of light, first with a Philips Master TL-D 36 W and later with a Philips Master HPI-T Plus 400 W. Upon reaching an appropriate size, the plants were transferred to 10 L pots and exposed to a flowering light cycle of 12 h of light (Philips Master Green Power Plus 600 W) until harvest. The soil used was Subtract 144 from Ricoter (Aarberg, Switzerland), and the nutrient used was Plantactiv 18+12+18 Type A from Hauert (Grossaffoltern, Switzerland). Each week, some plants were cut, dried for 1 week at 20 °C and 45% humidity, and analyzed to determine their cannabinoid and terpene contents.

Extraction. Cannabis plant materials (0.1 g) were weighed in a glass vial, and after adding 1 mL of EtOH/$CHCl_3$ (9:1 ratio), the mixtures were sonicated for 15 min. The samples were subsequently filtered and diluted in EtOH at 1:100 for cannabinoid analysis and at 1:10 for terpene analysis. Internal standards were added in the dilution step. Phenanthrene was added to obtain a final concentration of 20 μg/mL for cannabinoids, and 1-octanol was added to obtain a final concentration of 100 μg/mL for terpenes. The samples were injected just after preparation.

Standards and Materials. Reference cannabinoids THCA, THC, CBD, and CBN were purchased from Lipomed (Arlesheim, Switzerland), and CBDA, CBGA, CBG, and CBC were purchased from Echo Pharmaceuticals BV (Weesp, The Netherlands). Reference terpenes α-pinene, β-pinene, myrcene, limonene, eucalyptol, terpinolene, linalool, β-caryophyllene, humulene, and guaiol, internal standards phenanthrene and 1-octanol, and ≥99% HOAc were obtained from Sigma-Aldrich (Steinheim, Germany). HPLC-quality MeOH, EtOH, $CHCl_3$, and H_2O were purchased from Carl Roth (Karlsruhe, Germany). Nitrogen, hydrogen, helium, and synthetic air of 99.999% purity were obtained from Carbagas (Lausanne, Switzerland).

Data Analysis and Statistics. On the basis of the terpene and cannabinoid concentrations, a multivariate data analysis was performed to identify the characteristic terpenes for each chemotype. PCA and PLS were accomplished with the statistical software package The Unscrambler (9.7 Camo Asa, Oslo, Norway) to identify specific patterns in the study design and to build regression models against the maturation time. PCA uses an orthogonal transformation to transform a number of possibly correlated variables into linearly uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the matrix data as possible, and the next principal component accounts for as much of the remaining variability as possible. Thus, the dimensionality of the data set can be reduced, and the underlying variables can be identified. Moreover, cross-correlation was used to evaluate how the compounds were correlated with each other during plant growth. PLS employs a similar strategy, but instead of finding the PCs that minimize the variance between the response and independent variables, the algorithm maximizes the correlation of each PC with the dependent variables (i.e., the time).

Concentration data [x (224 samples × 36 variables) and y (224 samples × 1 variable (time)], including categorical data, such as the type of plant, the phase of plant development, and the analysis of leaves or flowers, were uploaded in The Unscrambler. Because in most of the cases the concentrations of the cannabinoids and terpenes ranged from values lower than the detection limits at the early development stage, the distribution of the concentrations was not normal, and there were many values < LOD or missing values. Thus, the data were transformed to $\log(x + 0.005)$ to eliminate the values < LOD and to improve the normality of the distribution.

Initial PCA and PLS models were built with standardized x variables to give equal weight to all variables and make use of the leverage correction method as the internal validation procedure. Once the putative outliers were removed and a robust model was obtained, a final model was built making use of the cross-correlation as the internal validation procedure.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00949.

Additional details (PDF)

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Notes

The authors declare no competing financial interest.

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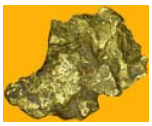

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resin

Soft solid or highly viscous substance, usually containing prepolymers with reactive groups.
Notes:

1. This term was used originally because of its analogy with a natural resin (rosin) and designated, in a broad sense, any used in a more narrow sense to refer to prepolymers of thermosets (thermosetting polymers).
2. The term is sometimes used not only for prepolymers of thermosets, but also for cured thermosets (e.g., epoxy resins,
3. Use of the term 'resin' to describe the polymer beads used in solid-phase synthesis and as polymer supports, catalysts

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		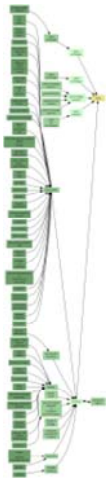

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