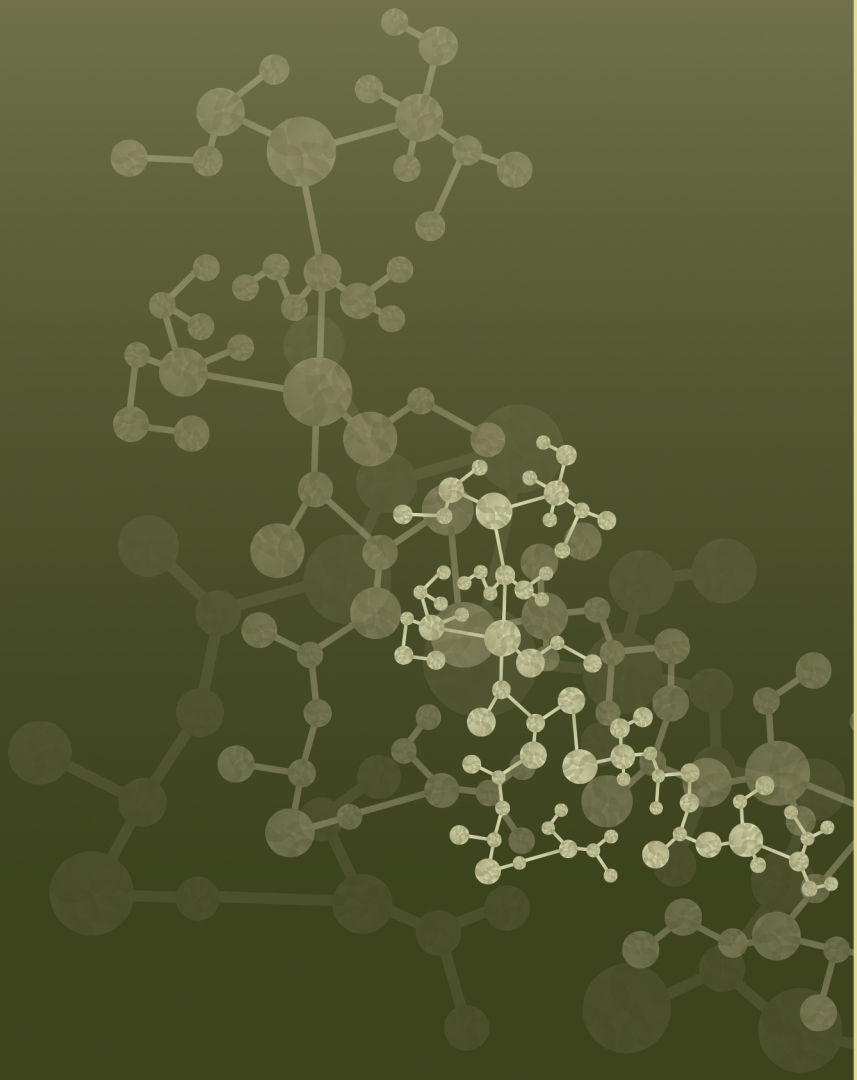
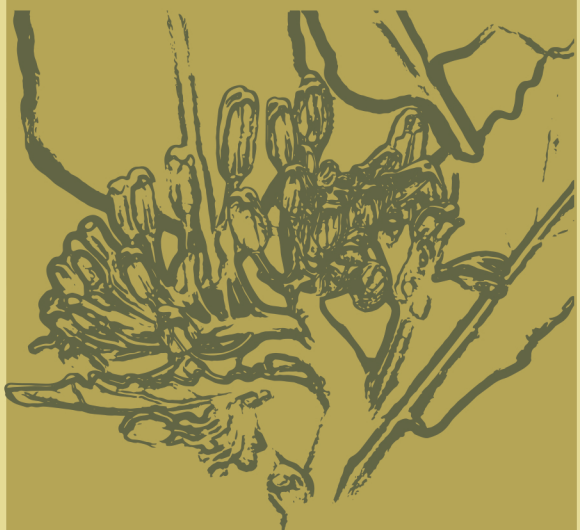
Brassica oleracea como fuente de compuestos biosaludables: aproximación genética



Tamara Sotelo Pérez

Tesis Doctoral

2015



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BRASSICA OLERACEA COMO FUENTE DE COMPUESTOS BIOSALUDABLES: APROXIMACIÓN GENÉTICA

Memoria presentada por **Tamara Sotelo Pérez**
para la obtención del grado de Doctor con Mención Internacional por la Universidad de Vigo.

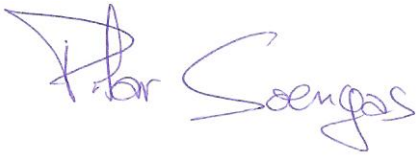
Trabajo presentado por Tamara Sotelo Pérez para la obtención del grado de Doctor con Mención Internacional por la Universidad de Vigo

Fdo.: Tamara Sotelo Pérez
Pontevedra, enero de 2015

Directores:

Mª Elena Cartea González
Investigadora Científica
Misión Biológica de Galicia (CSIC)
Pontevedra

Pilar Soengas Fernández
Científica Titular
Misión Biológica de Galicia (CSIC)
Pontevedra



Fdo.: Elena Cartea González

Fdo.: Pilar Soengas Fernández

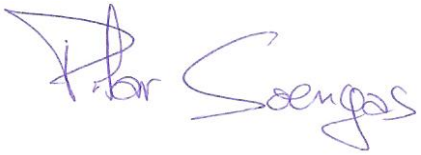
Pontevedra, enero de 2015

Dña. Mª Elena Cartea González, Investigadora Científica del Consejo Superior de Investigaciones Científicas y Dña. Pilar Soengas Fernández, Científica titular del Consejo Superior de Investigaciones Científicas, ambas investigadoras de la Misión Biológica de Galicia, en Pontevedra

INFORMAN:

Que el trabajo titulado "*Brassica oleracea* como fuente de compuestos biosaludables: aproximación genética" realizado por la licenciada en Biología Dña. Tamara Sotelo Pérez, bajo la dirección de las Dras. Elena Cartea González y Pilar Soengas Fernández, puede ser presentado para su exposición y defensa como Tesis Doctoral para la obtención el grado de Doctor con Mención Internacional en el Programa Oficial de Doctorado Ecosistemas Terrestres, Uso Sostenible e implicaciones Ambientales de la Universidad de Vigo.

Considerando que se encuentra concluida damos el Vº Bº para su presentación y lectura.



Fdo.: Elena Cartea González **Fdo.: Pilar Soengas Fernández**

Pontevedra, enero de 2015

A mis padres

"Esto no es el final. Ni tampoco es el principio del final.
Pero quizás sea, el final del principio"

W. Churchill

"Nunca tenga certeza de nada,
porque la sabiduría comienza con la duda"

Freud

AGRADECIMIENTOS

Es difícil entender la importancia de los agradecimientos de una Tesis Doctoral hasta que no se ha terminado. En ese momento te das cuenta de cuánto tienes que agradecer a tanta gente. Considero que todo aquel que ha tenido alguna relación conmigo durante el desarrollo de esta tesis merece mi total agradecimiento, ya que estoy segura de que en algún momento ha escuchado estoicamente quejas y lamentaciones, alegrías y satisfacciones así que intentaré resumir en unas líneas la gratitud que siento hacia todas las personas que han estado presentes durante esta etapa, haciendo posible que hoy deje de ser un sueño para pasar a ser una realidad.

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A mis amigos, los de siempre y los recientes, que han sabido disculpar mis ausencias y siempre han tenido una palabra de ánimo. Estoy absolutamente convencida

de que si de algo puedo presumir en esta vida es de los grandes amigos que tengo, lo que me hace sentir una persona muy afortunada. No hace falta que los nombre, ellos saben quiénes son y lo importantes que son para mí y, aunque algunos están lejos, tengo la suerte de poder sentirme siempre acompañada.

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A todos mi eterno agradecimiento.

Universidade Vigo

BRASSICA OLERACEA COMO FUENTE DE COMPUESTOS BIOSALUDABLES: APROXIMACIÓN GENÉTICA

Memoria realizada para optar al grado de Doctor con Mención Internacional por la Universidad de Vigo

TAMARA SOTELO PÉREZ

2015

RESUMEN

El género *Brassica* pertenece a la familia *Brassicaceae* y está integrado por un gran número de especies y cultivos con una gran diversidad de características morfológicas. Actualmente este género está formado por 37 especies, las cuales tienen usos tan variables como la alimentación humana y animal, la industria o la ornamentación. Este género presenta además una gran variabilidad tanto de formas como de productos; sin embargo, la importancia económica se centra de manera casi exclusiva en seis especies. Una de estas especies es *Brassica oleracea*, en la cual se engloban los cultivos agrícolas de mayor distribución mundial de este género. Esta especie limita su aprovechamiento a los tejidos epicotilares vegetativos (berzas, repollos…) y fructíferos (brécol y coliflor). La mayoría de los cultivos de *B. oleracea* tienen un uso hortícola y sus partes verdes se consumen en crudo o cocinados siendo el ingrediente de muchos platos tradicionales, como el cocido en España, el caldo verde en Portugal o el chucrut en Alemania. *B. oleracea* también está presente en la alimentación animal pudiendo utilizarse como suplemento en forma de forraje, incluso es fácil verla como planta ornamental de exterior siendo habitual en zonas verdes decorando parques, parterres y bancales en los meses de invierno.

La relación existente entre los hábitos alimenticios y el desarrollo de ciertas dolencias ha impulsado el incremento de una conciencia social hacia la mejora de la alimentación, así como de las instituciones gubernamentales para desarrollar campañas dirigidas a estimular el consumo de alimentos de origen vegetal y llevar una alimentación más saludable como medida preventiva frente al desarrollo de enfermedades. Un gran número de estudios epidemiológicos ha demostrado que un consumo regular de vegetales en general y, de brásicas en particular (grelos, berzas, repollo, coliflor o brécol, entre otros), ayuda a la disminución del riesgo de padecer diferentes tipos de cáncer y otras enfermedades crónicas, incidiendo favorablemente en la mejora de la calidad de vida de las personas. Este efecto beneficioso para la salud humana ha sido atribuido, en parte, a la presencia de ciertos metabolitos secundarios denominados compuestos bioactivos que, aunque en un principio se clasificaron como simples productos de desecho, actualmente se les considera importantes por sus numerosas y complejas funciones biológicas, además de tener un impacto significativo

en la salud humana. En los últimos años, estos compuestos bioactivos han sido objeto de una importante labor de investigación sobre sus propiedades, absorción, metabolismo y actividad biológica, aunque es necesario llevar a cabo estudios más exhaustivos para comprender los mecanismos de su síntesis, así como de su acción sobre la salud humana.

Entre los compuestos bioactivos presentes en los cultivos de brásicas destacan de modo especial los glucosinolatos (GSLs) y diversos compuestos con actividad antioxidante, entre los que se incluyen los carotenoides y compuestos fenólicos. En los últimos años, se ha demostrado que la ingesta de estos compuestos ayuda a proteger a las células contra el daño oxidativo, induce la detoxificación de enzimas, estimula el sistema inmune y reduce el riesgo de padecer enfermedades degenerativas o cardiovasculares. Además, los compuestos citados tienen una función defensiva en la planta frente a patógenos y plagas y frente a estreses abióticos como el frío o el exceso de radiación ultravioleta. Por lo tanto, la concentración y el contenido de estos compuestos, se han convertido en caracteres de especial interés a la hora de incluirlos en programas de mejora vegetal, con el fin de obtener cultivos enriquecidos y de mayor valor añadido.

Los cultivos del género *Brassica* destacan por su alto contenido en compuestos antioxidantes. La capacidad antioxidante de las brásicas es alta comparada con la de otras hortalizas; de hecho, el brécol y las coles destacan entre otros cultivos superando a la espinaca o a la zanahoria, entre otros. Debido a la complejidad de la composición en antioxidantes de los distintos extractos vegetales y de las diferentes interacciones sinérgicas entre los diferentes compuestos, la separación y estudio de cada compuesto individual son muy costosos e ineficientes. Por todo ello se ha desarrollado un amplio número de métodos para evaluar la capacidad antioxidante de los extractos vegetales *in vitro.* Los métodos ABTS, FRAP y DPPH, se emplean comúnmente en estudios sobre cultivos de brásicas y podrían ser utilizados como una herramienta dentro de distintos programas de mejora. Además, debido a la alta correlación observada entre estos métodos podría ser posible utilizar uno solo de ellos para evaluar la capacidad antioxidante de un cultivo.

Se sabe que determinados compuestos, como los fenólicos o carotenoides, confieren capacidad antioxidante al organismo. La capacidad antioxidante de las

brásicas se relaciona en mayor medida con su composición en compuestos fenólicos. Dichos compuestos constituyen una de las principales clases de metabolitos secundarios presentes en las brásicas, donde intervienen en diversas funciones fisiológicas, como el crecimiento y reproducción de las plantas, la protección frente a patógenos y predadores o la radiación ultravioleta. Dentro del grupo de los compuestos fenólicos se encuentra uno de los grupos más importantes de pigmentos naturales, los antocianos. Gracias a su diversidad estructural y a su estructura química actúan como antioxidantes, donando hidrógenos y electrones a los radicales libres o bien atrapándolos en su estructura aromática. Los carotenoides son un grupo estructuralmente muy diverso y en él se incluyen más de 600 pigmentos naturales. Algunos de ellos, son nutrientes esenciales en la dieta humana mientras que otros muestran efectos positivos frente a diferentes enfermedades.

A pesar del uso cada vez más generalizado de los métodos de medida de la capacidad antioxidante, se desconoce su genética así como la de los compuestos con capacidad antioxidante. Por ello, en esta tesis se plantea llevar a cabo un análisis de QTLs como primer paso a la hora de estudiar el control genético de un carácter cuantitativo. Este estudio constituye la primera etapa para poder llegar a identificar los posibles genes candidatos responsables de esta variación.

Se considera también que los GSLs son compuestos antioxidantes, no porque tengan actividad antioxidante '*per se*', sino porque contribuyen a potenciar los sistemas enzimáticos antioxidantes de la célula. Estos compuestos representan el grupo más importante de metabolitos secundarios presentes en las brásicas debido a su singularidad ya que se encuentran en la naturaleza de manera exclusiva en las especies del orden *Brassicales*, en el que se incluye el género *Brassica*. Hasta la actualidad, se han identificado más de 200 GSLs distintos que difieren en las modificaciones sufridas en su cadena lateral. En función del aminoácido de procedencia se clasifican en alifáticos, indólicos y aromáticos.

La variación en la composición y concentración de los GSLs depende de distintos factores entre los que se encuentran tanto los factores genéticos como los ambientales. Además del genotipo, están involucrados el órgano de la planta, la etapa de desarrollo así como diversos factores post-cosecha y de procesado. Dentro de cada subespecie o tipo de brásicas, aparece el mismo patrón de GSLs, encontrándose

normalmente entre 10 y 12 GSLs distintos dentro de cada cultivo. Los GSLs mayoritarios en *B. oleracea* son los alifáticos y, en menor medida, los indólicos. En realidad, no son los GSLs intactos sino los productos de hidrólisis de los mismos los que suponen a la planta un sistema de defensa y son además los responsables de las propiedades beneficiosas para la salud atribuidas a estos compuestos. Diversos estudios han determinado que los mecanismos de acción de los compuestos derivados de los GSLs en la salud humana incluyen la modulación de enzimas implicadas en procesos de carcinogénesis, la protección frente al estrés oxidativo y la inhibición del desarrollo de tumores. Debido a todas estas propiedades, se están realizando importantes esfuerzos para localizar los principales genes responsables de la síntesis de GSLs en distintas especies del género *Brassica* y así conocer de modo exhaustivo las vías de síntesis y acumulación de estos metabolitos, lo que ofrece futuras posibilidades para poder manipular e incrementar el contenido de aquellos GSLs de interés y beneficiosos para la salud humana.

Hasta la actualidad, se han identificado algunos de los principales genes de la ruta de síntesis de los GSLs alifáticos; sin embargo, aún quedan por identificar otros genes responsables de las diferentes etapas de la ruta de biosíntesis de alifáticos, indólicos y aromáticos. Para la identificación de los genes implicados en la ruta de biosíntesis existen diferentes técnicas moleculares entre las que se encuentra el análisis de QTLs. A pesar de que *B. oleracea* es una de las especies más importantes desde el punto de vista económico dentro de su género, hasta la fecha este es el primer estudio de mapeo de QTLs para el contenido en GSLs en esta especie.

En muchos cultivos hortícolas, como los que se encuentran dentro de *B. oleracea*, los programas de mejora genética han atendido principalmente a aspectos como la productividad, resistencia a enfermedades, uniformidad del producto y calidad externa. Sin embargo, en los últimos años estos programas de mejora genética han ido incorporando la mejora del contenido en compuestos bioactivos beneficiosos para la salud humana dentro de sus objetivos debido al creciente interés por el consumo de productos vegetales sanos. De este modo, la modificación del contenido de los GSLs, se convierte en un nuevo objetivo dentro de la mejora de esta especie. El conocimiento de las bases genéticas de la síntesis y regulación de estos metabolitos, ofrece la posibilidad de manipular los perfiles de GSLs, incrementando aquellos con propiedades

beneficiosas para la salud humana y reduciendo otros con efectos perjudiciales. Hasta la fecha, la modificación del contenido de GSLs se había realizado mediante introgresiones a partir de germoplasma silvestre o mediante transformación genética, pero no se había utilizado un método de selección aprovechando la variabilidad natural existente dentro de los propios cultivos. Por ello, en esta tesis, se plantea la realización de una selección masal para el contenido en diferentes GSLs individuales en hojas, así como el estudio del efecto de esta selección en los diferentes órganos y sobre el resto de los GSLs presentes en la planta. Este método ha sido practicado por los agricultores a lo largo de los siglos para mejorar características deseables en diferentes cultivos y todavía se utiliza hoy en día en programas de mejora genética cuando es esencial la rapidez del proceso y siempre y cuando el carácter tenga una alta heredabilidad. Además, en el caso de los cultivos de *B. oleracea* es el método de selección más fácil de llevar a cabo debido al carácter alógamo de la especie y a la dificultad de realizar cruzamientos manuales.

Con estos antecedentes, el principal objetivo de esta tesis doctoral es ampliar el conocimiento sobre los mecanismos o los genes implicados en la síntesis y acumulación de distintos compuestos beneficiosos para la salud humana, como son los compuestos con actividad antioxidante y los GSLs presentes en *B. oleracea*. Se estudiará la relación entre los diferentes métodos para medir actividad antioxidante y su relación con el contenido en compuestos fenólicos, carotenoides y antocianinas. Se estudiarán y buscarán QTLs relacionados con los métodos de medida de la capacidad antioxidante, con los metabolitos relacionados con esta capacidad y con el contenido en compuestos GSLs como un paso previo a la identificación de posibles genes candidatos. Además, se determinará si las selecciones divergentes diseñadas para el contenido de los tres GSLs mayoritarios en hojas de berza han sido efectivas y paralelamente se estudiarán los cambios indirectos producidos al efectuar esta selección tanto en otros órganos de la planta como en la concentración del resto de GSLs.

En la presente tesis se ha llevado a cabo una comparación de tres métodos de medida de la capacidad antioxidante (FRAP, ABTS y DPPH) en una población de mapeo de dobles haploides (DH) en dos órganos diferentes de la planta (hojas y brotes florales). Los resultados mostraron la necesidad de aplicar más de un método de medida a la hora de evaluar de modo correcto la actividad antioxidante de una variedad, debido

a las bajas correlaciones existentes entre los diferentes métodos cuando se evalúa un material que está genéticamente relacionado, como el caso de las líneas DH estudiadas en esta tesis.

Se analizaron diferentes metabolitos que pueden ser los causantes de la actividad antioxidante, como antocianinas, compuestos fenólicos totales y carotenoides. En este caso, se encontraron correlaciones entre los distintos métodos de medida y el contenido de fenólicos totales y carotenoides, lo que confirmaría que estos compuestos son, en parte, los responsables de la capacidad antioxidante de las brásicas.

Se encontraron un total de 19 QTLs, de los cuales 9 fueron encontrados en hojas y 10 en brotes florales. Se detectaron regiones genómicas con QTLs para los métodos de medida y los metabolitos responsables de la capacidad antioxidante. La importancia de estos resultados radica en que hasta la fecha no se han publicado trabajos en brásicas orientados a la evaluación y comparación de distintos métodos de medida de capacidad antioxidante y que identifiquen QTLs relacionados dicha actividad.

Además de los compuestos antioxidantes, se llevó a cabo un estudio genético sobre los GSLs en tres órganos diferentes de las plantas, hojas, semillas y brotes florales en una población de DH de *B. oleracea*. Los resultados obtenidos en el estudio de la concentración de GSLs, muestran, al igual que estudios previos, que existe una mayor concentración de GSLs en los órganos reproductivos que en los vegetativos. De este modo, el mayor contenido de GSLs se encontraría en las semillas, seguido de los brotes florales y, por último, en las hojas. Con respecto a la búsqueda de QTLs y genes candidatos implicados en la ruta de biosíntesis de GSLs, se encontraron un total de 20 QTLs consenso, de los cuales dos fueron específicos de semillas, dos de brotes florales y uno de hojas.

La detección de QTLs específicos dentro de cada órgano tanto para el estudio de antioxidantes como en el de GSLs, supone que parte de la regulación de las rutas de síntesis es dependiente del órgano y, por lo tanto, sería posible obtener variedades enriquecidas en un determinado compuesto y en un determinado órgano dentro de la planta. Se encontraron tres regiones genómicas que controlan la variabilidad y el contenido de compuestos con capacidad antioxidante y GSLs en los cromosomas 3, 5 y

7. En las tres regiones genómicas el alelo que incrementa el carácter es aportado por el mismo padre.

Para ambos estudios de QTLs y, gracias al parentesco genético de la especie bajo estudio, *B. oleracea* y la planta modelo por excelencia *Arabidopsis thaliana*, se realizó un estudio de sintenia entre ambas especies para poder identificar los genes homólogos en *B. oleracea* que afectan tanto a los GSLs como a los métodos de medida de la capacidad antioxidante y a los metabolitos responsables de la misma. De hecho, se han podido localizar '*in silico'* genes relacionados con la ruta de síntesis de los compuestos fenólicos y de los GSLs en los intervalos de confianza de los QTLs encontrados a lo largo de esta tesis. Concretamente, en el estudio de antioxidantes, se identificaron algunos genes clave en la ruta de síntesis de los fenilpropanoides (ruta relacionada con la síntesis de los compuestos fenólicos) como el HCT y el C3'H en el cromosoma 3. Esta región se muestra como una zona de importancia relevante ya que en ella se han detectado también QTLs para los tres métodos de medida analizados (FRAP, ABTS y DPPH). Por otro lado, se ha logrado identificar los principales loci que controlan la mayor parte de la variabilidad de los GSLs alifáticos: GSL-OH, GSL-PRO y GSL-ALK. En el caso de los GSLs indólicos y aromáticos, se localizaron los genes CYP79B2, CYP81F2, CYP79B3 y ATR1.

Todos los genes candidatos podrán ser estudiados y corroborados en breve, ya que unos de los parentales de la población de mapeo empleada para los estudios de QTLs, TO1000DH3, ha sido utilizado en el estudio de secuenciación de *B. oleracea*, cuya secuencia (aunque todavía incompleta) acaba de ser publicada recientemente. Esta publicación permitirá en un futuro no muy lejano, corroborar los resultados encontrados, así como la búsqueda mucho más rápida y directa de otros genes implicados en la ruta de biosíntesis de los distintos metabolitos de interés.

Además de la identificación de QTLs y genes candidatos, es de vital importancia determinar las posibles relaciones epistáticas existentes entre los distintos loci identificados, las cuales podrían influir en gran medida en la variabilidad y en el contenido de los metabolitos de interés. En el estudio genético de GSLs, se detectó un elevado número de interacciones epistáticas entre los distintos QTLs, siendo algunas de ellas comunes a los tres órganos estudiados. De todas las relaciones encontradas, cabe destacar el papel del locus GSL-ALK en la determinación de la variación de GSLs

alifáticos. Este locus, ocupa el centro de una red de interacciones epistáticas en donde participan hasta 12 QTLs diferentes.

Para comprobar si la selección masal puede resultar un método eficaz para incrementar y/o disminuir el contenido de un GSL concreto y con el fin de obtener material con un contenido modificado en GSLs, se evaluaron tres selecciones divergentes en una población local de berzas (*B. oleracea* var. *acephala*). Se llevaron a cabo tres programas de selección, uno para cada GSL mayoritario presente en las hojas: dos GSLs alifáticos, sinigrina (SIN) y glucoiberina (GIB) y el tercero para el GSL indólico, glucobrasicina (GBS). Los resultados mostraron que es posible modificar cuantitativamente la concentración de los GSLs citados en las hojas sobre un mismo fondo genético, indicando que la concentración de GSLs es un carácter altamente heredable y que puede modificarse mediante métodos de selección convencional. La respuesta se ajusta a un modelo de regresión lineal y no hay signos de que en las hojas, la variabilidad genética para este carácter se haya agotado tras tres ciclos de selección en ninguna de las direcciones, por lo que cabría la posibilidad de desarrollar nuevos ciclos de selección con éxito.

Además, se evaluó el efecto de esta modificación de GSLs realizada en las hojas en la composición y concentración de estos mismos GSLs en los brotes florales y semillas, encontrando un efecto secundario de las selecciones sobre estos órganos. Esto indicaría que existe una modificación en la síntesis de GSLs en los brotes florales y semillas o a una translocación de los GSLs sintetizados en las hojas a estos otros dos órganos.

En tercer lugar, se comprobó que el hecho de alterar la concentración de un GSL en particular tiene un efecto sobre el resto de GSLs de la planta ya sea en mayor o menor medida, principalmente en aquellos implicados en la misma ruta de síntesis. Los efectos indirectos observados en las selecciones de los dos GSLs alifáticos (SIN y GIB) sobre el resto de GSLs de la planta sugieren que los diferentes alelos del locus GSL-ALK podrían ser los responsables de la variación de la concentración a través de los ciclos de selección. Además, los resultados apoyan también la hipótesis de que el locus GSL-ALK controla indirectamente la variabilidad del GSL indólico GBS, indicando un cruce o relación entre ambas rutas de biosíntesis.

Al comparar los dos trabajos de GSLs llevados a cabo en esta tesis (búsqueda de genes y modificación en la composición mediante selección) observamos que, aunque son estudios independientes, el locus GSL-ALK se postula como el responsable de gran parte de la variación observada en los GSLs alifáticos. Por ello, es obvio que este locus debe tenerse en cuenta en los programas de mejora destinados a modificar el contenido en GSLs, si bien es preciso sopesar que la selección por este locus podría tener efectos indirectos sobre otros genes de la ruta de síntesis de estos metabolitos.

Como conclusión, los datos aportados en este trabajo pueden ser de gran utilidad para profundizar en el conocimiento acerca de la síntesis y regulación de los compuestos biosaludables en los cultivos de *B. oleracea*.

RESUMO

O xénero *Brassica* pertence á familia *Brassicaceae* e está integrado por un gran número de especies e cultivos cunha gran diversidade de características morfolóxicas. Actualmente este xénero está formado por 37 especies, as cales teñen usos tan variables como a alimentación humana e animal, a industria ou a ornamentación. Este xénero presenta ademais unha gran variabilidade tanto de formas coma de produtos; non obstante, a importancia económica céntrase de xeito case exclusivo en seis especies. Unha destas especies é *Brassica oleracea*, na cal se engloban os cultivos agrícolas de maior distribución mundial deste xénero. Esta especie limita o seu aproveitamento aos tecidos epicotilares vexetativos (verzas, repolos...) e frutíferos (brócoli e coliflor). A maioría dos cultivos de *B. oleracea* teñen un uso hortícola e as súas partes verdes consúmense tanto en cru coma cociñados sendo o ingrediente de moitos pratos tradicionais, como o cocido en España, o caldo verde en Portugal ou o chucrut en Alemaña. *B. oleracea* tamén está presente na alimentación animal pudendo utilizarse como suplemento en forma de forraxe, mesmo é doado vela como planta ornamental de exterior sendo habitual en zonas verdes decorando parques, xardíns e bancais nos meses de inverno.

A relación existente entre os hábitos alimenticios e o desenvolvemento de certas doenzas impulsou o incremento dunha conciencia social cara á mellora da alimentación, así como das institucións gobernamentais para desenvolver campañas dirixidas a estimular o consumo de alimentos de orixe vexetal e levar unha alimentación máis saudable como medida preventiva fronte ao desenvolvemento de enfermidades. Un gran número de estudos epidemiolóxicos demostraron que un consumo regular de vexetais en xeral e, en particular de brásicas (grelos, verzas, repolo, coliflor ou brócoli, entre outros), axuda á diminución do risco de padecer diferentes tipos de cancro e outras enfermidades crónicas, incidindo favorabelmente na mellora da calidade de vida das persoas. Este efecto beneficioso para a saúde humana foi atribuído, en parte, á presenza de certos metabolitos secundarios denominados compostos bioativos, aínda inicialmente clasificados como simples produtos de refugallo, son agora considerados importantes polas súas numerosas e complexas funcións biolóxicas, ademais de ter un impacto significativo na saúde humana. Nos últimos anos, estes compostos bioativos foron

obxecto dun importante labor de investigación sobre as súas propiedades, absorción, metabolismo e actividade biolóxica, aínda que é necesario levar a cabo estudos máis exhaustivos para comprender os mecanismos da súa síntese, así como da súa acción sobre a saúde humana.

Entre os compostos bioativos presentes nos cultivos de brásicas destacan de modo especial os glicosinolatos (GSLs) e diversos compostos con actividade antioxidante, entre os que se inclúen os carotenoides e compostos fenólicos. Nos últimos anos, demostrouse que a inxestión destes compostos axuda a protexer as células contra o dano oxidativo, induce a detoxificación de enzimas, estimula o sistema inmune, e reduce o risco de padecer enfermidades dexenerativas ou cardiovasculares. Ademais, os compostos citados teñen unha función defensiva na planta fronte a patóxenos e pragas e fronte a estreses abióticos como o frío ou o exceso de radiación ultravioleta. Polo tanto, a concentración e o contido destes compostos, convertésense en carácteres de especial interese á hora de incluílos en programas de mellora vexetal, co fin de obter cultivos enriquecidos e de maior valor engadido.

Os cultivos do xénero *Brassica* destacan polo seu alto contido en compostos antioxidantes. A actividade antioxidante das brásicas é alta comparada coa doutras legumes; de feito, o brócoli e as coles destacan entre outros cultivos superando á espinaca ou á cenoria, entre outros. Debido á complexidade da composición en antioxidantes dos distintos extractos vexetais e das diferentes interaccións sinérxicas entre os diferentes compostos, a separación e estudo de cada composto individual é moi custoso e ineficiente. Por todo iso desenvolvéronse un amplo número de métodos para avaliar a actividade antioxidante dos extractos vexetais *in vitro*. Os métodos ABTS, FRAP e DPPH, comunmente empregados en estudos sobre cultivos de brásicas poderían ser utilizados como unha ferramenta dentro de distintos programas de mellora. Ademais, debido á correlación observada entre estes métodos podería ser posible utilizar un só deles para avaliar a actividade antioxidante dun cultivo.

Sábese que determinados compostos, como os fenólicos ou carotenoides, confiren actividade antioxidante ao organismo. A capacidade antioxidante das brásicas relaciónase en maior medida coa súa composición en compostos fenólicos. Os devanditos compostos constitúen unha das principais clases de metabolitos secundarios presentes nas brásicas, onde interveñen en diversas funcións fisiolóxicas,

como o crecemento e reprodución das plantas, a protección fronte a patóxenos e predadores ou a radiación ultravioleta. Dentro do grupo dos compostos fenólicos encóntranse un dos grupos máis importantes de pigmentos naturais, os antocianos. Grazas á súa diversidade estrutural e á súa estrutura química actúan como antioxidantes, doando hidróxenos e electróns aos radicais libres ou ben atrapándoos na súa estrutura aromática. Os carotenoides é un grupo estruturalmente moi diverso e nel inclúense máis de 600 pigmentos naturais. Algúns deles, son nutrientes esenciais na dieta humana mentres que outros mostran efectos positivos fronte a diferentes enfermidades.

A pesar do uso cada vez máis xeneralizado dos métodos de medida da actividade antioxidante, descoñécese a súa xenética así como a dos compostos con capacidade antioxidante. Por iso, nesta tese xorde levar a cabo unha análise de QTLs como primeiro paso á hora de estudar o control xenético dun carácter cuantitativo. Este estudo constitúe a primeira etapa para poder chegar a identificar os posibles xenes candidatos responsables desta variación.

Considérase tamén que os GSLs son compostos antioxidantes, non porque teñan actividade antioxidante '*per se*', senón porque contribúen a potenciar os sistemas enzimáticos antioxidantes da célula. Estes compostos representan o grupo máis importante de metabolitos secundarios presentes nas brásicas debido á súa singularidade xa que se encontran na natureza de xeito exclusivo nas especies da orde Brassicales, no que se inclúe o xénero *Brassica*. Ata o momento, identificáronse máis de 200 GSLs distintos que difiren nas modificacións sufridas na súa cadea lateral. En función do aminoácido de procedencia clasifícanse en alifáticos, indólicos e aromáticos.

A variación na composición e concentración dos GSLs depende de distintos factores entre os que se encontran factores xenéticos e ambientais. Ademais do xenotipo, están involucrados o órgano da planta, a etapa de desenvolvemento así como diversos factores post-colleita e de procesamento. Dentro de cada subespecie ou tipo de brásicas, aparece o mesmo patrón de GSLs, encontrándose normalmente entre 10 e 12 GSLs distintos dentro de cada cultivo. Os GSLs maioritarios en *B. oleracea* son os alifáticos e, en menor medida, os indólicos. De feito, non son os GSLs intactos senón os produtos de hidrólise destes os que supoñen á planta un sistema de defensa e son ademais os responsables das propiedades beneficiosas para a saúde atribuídas a estes compostos. Diversos estudos determinaron que os mecanismos de acción dos compostos

derivados dos GSLs na saúde humana inclúen a modulación de enzimas implicadas en procesos de carcinoxénese, a protección fronte ao estrés oxidativo e a inhibición do desenvolvemento de tumores. Debido a todas estas propiedades, estanse a realizar importantes esforzos para localizar os principais xenes responsables da síntese de GSLs en distintas especies do xénero *Brassica* e así coñecer de modo exhaustivo as vías de síntese e acumulación destes metabolitos, o que ofrece futuras posibilidades para poder manipular e incrementar o contido daqueles GSLs de interese e beneficiosos para a saúde humana.

Ata o momento, identificáronse algúns dos principais xenes da ruta de síntese dos GSLs alifáticos; con todo, aínda quedan por identificar outros xenes responsables das diferentes etapas da ruta de biosíntese de alifáticos, indólicos e aromáticos. Para a identificación dos xenes implicados na ruta de biosíntese existen diferentes técnicas moleculares entre as que se encontra a análise de QTLs. A pesar de que *B. oleracea* é unha das especies máis importantes dende o punto de vista económico dentro do seu xénero, ata a data este é o primeiro estudo de cartografía de QTLs para o contido en GSLs nesta especie.

En moitos cultivos hortícolas, como os que se encontran dentro de *B. oleracea*, os programas de mellora xenética atenderon principalmente a aspectos como a produtividade, resistencia a enfermidades, uniformidade do produto e calidade externa. Non obstante, nos últimos anos estes programas de mellora xenética foron incorporando a mellora do contido en compostos bioativos beneficiosos para a saúde humana dentro dos seus obxectivos debido ao crecente interese polo consumo de produtos vexetais sans. Deste xeito, a modificación do contido dos GSLs, convértese nun novo obxectivo dentro da mellora desta especie. O coñecemento das bases xenéticas da síntese e regulación destes metabolitos, ofrece a posibilidade de manipular os perfís de GSLs, incrementando aqueles con propiedades beneficiosas para a saúde humana e reducindo outros con efectos prexudiciais. Ata a data, a modificación do contido de GSLs realizárase mediante introgresións a partir de xermoplasma silvestre ou mediante transformación xenética, pero non se utilizara un método de selección aproveitando a variabilidade natural existente dentro dos propios cultivos. Polo tanto, nesta tese, levouse a cabo a realización dunha selección masal para o contido en diferentes GSLs individuais nas follas, así como o estudo do efecto desta selección nos diferentes

órganos e sobre os demais GSLs presentes na planta. Este método foi practicado polos agricultores ao longo dos séculos para mellorar características desexables en diferentes cultivos e aínda se utiliza hoxe en día en programas de mellora xenética cando é esencial a rapidez do proceso e sempre e cando o carácter teña unha alta herdabilidade. Ademais, no caso dos cultivos de *B. oleracea* é o método de selección máis doado de levar a cabo debido ao carácter alógamo da especie e á dificultade de realizar cruzamentos manuais.

Con estes antecedentes, o principal obxectivo desta tese doutoral é ampliar o coñecemento sobre os mecanismos ou os xenes implicados na síntese e acumulación de distintos compostos beneficiosos para a saúde humana, como son os compostos con actividade antioxidante e os GSLs presentes en *B. oleracea*. Estudarase a relación entre os diferentes métodos para medir actividade antioxidante e a súa relación co contido en compostos fenólicos, carotenoides e antocianinas. Estudaranse e buscaranse QTLs relacionados cos métodos de medida da actividade antioxidante, cos metabolitos relacionados con esta capacidade e co contido en compostos GSLs como un paso previo á identificación de posibles xenes candidatos. Ademais, determinarase se as seleccións diverxentes deseñadas para o contido dos tres GSLs maioritarios nas follas de verza foron efectivas e paralelamente estudaranse os cambios indirectos producidos ao facer esta selección tanto noutros órganos da planta coma na concentración do resto de GSLs.

Na presente tese levouse a cabo unha comparación de tres métodos de medida da actividade antioxidante (FRAP, ABTS e DPPH) nunha poboación de cartografía de dobres haploides (DH) en dous órganos diferentes da planta (follas e brotes florais). Os resultados mostraron a necesidade de aplicar máis dun método de medida á hora de avaliar de modo correcto a actividade antioxidante dunha variedade, debido ás baixas correlacións existentes entre os diferentes métodos cando se avalía un material que está xeneticamente relacionado, como o caso das liñas DH estudadas nesta tese.

Analizáronse diferentes metabolitos que poden ser os causantes da actividade antioxidante, como antocianinas, compostos fenólicos totais e carotenoides. Neste caso, encontráronse correlacións entre os distintos métodos de medida e o contido de fenólicos totais e carotenoides, o que confirmaría que estes compostos son, en parte, os responsables da actividade antioxidante das brásicas.

Encontráronse un total de 19 QTLs, dos cales 9 encontráronse en follas e 10 en brotes florais. Detectáronse rexións xenómicas con QTLs para os métodos de medida e os metabolitos responsables da actividade antioxidante. A importancia destes resultados radica en que ata a data non se publicaron traballos en brásicas orientados á avaliación e comparación de distintos métodos de medida de actividade antioxidante e que identifiquen QTLs relacionados coa devandita actividade.

Ademais dos compostos antioxidantes, levouse a cabo un estudo xenético sobre, os GSLs en tres órganos diferentes das plantas, follas, sementes e brotes florais nunha poboación de DH de *B. oleracea*. Os resultados obtidos no estudo da concentración de GSLs, mostran, ao igual que os estudos previos, que existe unha maior concentración de GSLs nos órganos reprodutivos que nos vexetativos. Deste xeito, o maior contido de GSLs encontraríase nas sementes, seguido dos brotes florais e, por último, nas follas. En relación á busca de QTLs e xenes candidatos implicados na ruta de biosíntese de GSLs, encontráronse un total de 20 QTLs consenso dos cales dous foron específicos de sementes, dous de brotes florais e un de follas.

A detección de QTLs específicos dentro de cada órgano tanto para o estudo de antioxidantes coma no de GSLs, supón que parte da regulación das rutas de síntese é dependente do órgano e, polo tanto, sería posible obter variedades enriquecidas nun determinado composto e nun determinado órgano dentro da planta. Encontráronse tres rexións xenómicas que controlan a variabilidade e o contido de compostos con actividade antioxidante e GSLs nos cromosomas 3, 5 e 7. Nas tres rexións xenómicas o alelo que incrementa o carácter é achegado polo mesmo pai.

Para ambos os dous estudos de QTLs e, grazas ao parentesco xenético da especie baixo estudo, *B. oleracea* e a planta modelo por excelencia *Arabidopsis thaliana*, realizouse un estudo de sintenia entre ambas as dúas especies para poder identificar os xenes homólogos en *B. oleracea* que afectan tanto aos GSLs coma aos métodos de medida da actividade antioxidante e aos metabolitos responsables desta. De feito, puidéronse localizar '*in silico*' xenes relacionados coa ruta de síntese dos compostos fenólicos e dos GSLs nos intervalos de confianza dos QTLs encontrados ao longo desta tese. Concretamente, no estudo de antioxidantes, identificáronse algúns xenes clave na ruta de síntese dos fenilpropanoides (ruta relacionada coa síntese dos compostos fenólicos) como o HCT e o C3'H no cromosoma 3. Esta rexión móstrase como unha

zona de importancia relevante xa que nela se detectaron tamén QTLs para os tres métodos de medida analizados (FRAP, ABTS e DPPH). Por outro lado, logrouse identificar os principais loci que controlan a maior parte da variabilidade dos GSLs alifáticos: GSL-OH, GSL-PRO e GSL-ALK. No caso dos GSLs indólicos e aromáticos, localizáronse os xenes CYP79B2, CYP81F2, CYP79B3 e ATR1.

Todos os xenes candidatos poderán ser estudados e corroborados en breve, xa que uns dos parenterais da poboación de cartografía empregada para os estudos de QTLs, TO1000DH3, foi utilizado no estudo de secuenciación de *B. oleracea*, cuxa secuencia (aínda que incompleta) acaba de ser publicada recentemente. Esta publicación permitirá nun futuro non moi afastado, corroborar os resultados encontrados, así como a busca moito máis rápida e directa doutros xenes implicados na ruta de biosíntese dos distintos metabolitos de interese.

Ademais da identificación de QTLs e xenes candidatos, é de vital importancia determinar as posibles relacións epistáticas existentes entre os distintos loci identificados, as cales poderían influír en boa medida na variabilidade e no contido dos metabolitos de interese. No estudo xenético de GSLs, detectouse un elevado número de interaccións epistáticas entre os distintos QTLs, sendo algunhas delas comúns aos tres órganos estudados. De todas as relacións encontradas, cabe destacar o papel do locus GSL-ALK na determinación da variación de GSLs alifáticos. Este locus, ocupa o centro dunha rede de interaccións epistáticas onde participan ata 12 QTLs diferentes.

Para comprobar se a selección masal pode resultar un método eficaz para incrementar e/ou diminuír o contido dun GSL concreto e co fin de obter material cun contido modificado en GSLs, avaliáronse tres seleccións diverxentes nunha poboación local de verzas (*B. oleracea* var. *acephala*). Leváronse a cabo tres programas de selección, un para cada GSL maioritario presente nas follas: dous GSLs alifáticos, sinigrina (SIN) e glucoiberina (GIB) e o terceiro para o GSL indólico, glucobrasicina (GBS). Os resultados mostraron que é posible modificar cuantitativamente a concentración dos GSLs citados nas follas sobre un mesmo fondo xenético, indicando que a concentración de GSLs é un carácter altamente herdable e que pode modificarse mediante métodos de selección convencional. A resposta axústase a un modelo de regresión lineal e non hai sinais de que nas follas, a variabilidade xenética para este

carácter se esgotase tras tres ciclos de selección en ningunha das direccións, polo que cabería a posibilidade de desenvolver novos ciclos de selección con éxito.

Ademais, avaliouse o efecto desta modificación de GSLs realizada nas follas na composición e concentración destes mesmos GSLs nos brotes florais e sementes, encontrando un efecto secundario das seleccións sobre estes órganos. Isto indicaría que existe unha modificación na síntese de GSLs nos brotes florais e sementes ou a unha translocación dos GSLs sintetizados nas follas a estes outros dous órganos.

En terceiro lugar, comprobouse que o feito de alterar a concentración dun GSL en particular ten un efecto sobre o resto de GSLs da planta, xa sexa en maior ou menor medida, principalmente naqueles implicados na mesma ruta de síntese. Os efectos indirectos observados nas seleccións dos dous GSLs alifáticos (SIN e GIB) sobre o resto de GSLs da planta suxiren que os diferentes alelos do locus GSL-ALK poderían ser os responsables da variación da concentración a través dos ciclos de selección. Ademais, os resultados apoian tamén a hipótese de que o locus GSL-ALK controla indirectamente a variabilidade do GSL indólico GBS, indicando un cruzamento ou relación entre ambas as dúas rutas de biosíntese.

Ao comparar os dous traballos de GSLs levados a cabo nesta tese (busca de xenes e modificación na composición mediante selección) observamos que, aínda que son estudos independentes, o locus GSL-ALK postúlase como o responsable de gran parte da variación observada nos GSLs alifáticos. Por iso, é obvio que este locus debe terse en conta nos programas de mellora destinados a modificar o contido en GSLs, se ben é preciso sopesar que a selección por este locus podería ter efectos indirectos sobre outros xenes da ruta de síntese destes metabolitos.

Como conclusión, os datos achegados neste traballo poden ser de grande utilidade para afondar no coñecemento acerca da síntese e regulación dos compostos biosaludables nos cultivos de *B. oleracea*.

ABSTRACT

The *Brassica* genus belongs to the *Brassicaceae* family and is constituted by a high number of species and cultivars with a broad diversity of morphological characteristics. This genus is currently formed by 37 species with diverse uses such as human food, livestock fodder, industry or ornamentation. Species within this genus have a remarkable variability of morphologies and products; however, six species are the most important from an economic point of view. One of these species is *Brassica oleracea* which is worldwide distributed. Two organs have economic relevance in this species, the vegetative leaves (cabbage, kale…) and reproductive tissues (broccoli and cauliflower). Most of these crops are used for human consumption being the ingredient of traditional meals, such as "cocido" in Spain, "caldo verde" in Portugal or "chucrut" in Germany. Alternative uses of *B. oleracea* could be as fodder for livestock feeding or even as ornamental plants in gardens or terraces during winter.

The well-known relationship between food and the development of certain diseases has increased the social consciousness to improve food habits. It also stimulates governmental institutions to develop campaigns to encourage the consumption of vegetables for preventing the development of certain diseases. A high number of studies have demonstrated that a regular consumption of vegetables in general and, brassicas in particular, (turnip tops, kale, cabbage, cauliflower or broccoli, among others) diminishes the risk of developing certain cancers and other chronic diseases, increasing life quality. Health benefits of these crops have been attributed to certain secondary metabolites named bioactive compounds, which were initially considered waste compounds but currently are considered important due to their numerous and complex biological functions, besides of their important effect in the human health. During the last years, these bioactive compounds have been the focus of numerous research about their biological properties, absorption, metabolism and biological activity, although is necessary to carry out more exhaustive studies to understand the mechanism of their synthesis and their activity in the human health.

Among the active compounds present in the brassica crops, the most important are the glucosinolates (GSLs) and other compounds with antioxidant activity, which include carotenoids and phenolic compounds. In the last decades, it has been

demonstrated that the intake of these compounds could prevent the cellular oxidative damage, induce enzyme detoxification, stimulates the immune system and reduce the risk of developing degenerative or cardiovascular diseases. Likewise, these compounds have a plant defensive role against pathogens and pests and also against abiotic stresses such as cold or excess of UV radiation. Therefore, the content and concentration of these compounds are two traits of special relevance to be included in breeding programs to obtain enriched crops with a higher added value.

Brassica crops outstand due to their high content in antioxidant compounds. The antioxidant capacity of these crops is high compared to the other vegetables; indeed, broccoli and kales have a higher content of these compounds than that observed in spinach or carrots. Due to the complexity in the composition of antioxidant compounds in plant extracts and to the synergic interactions among the different compounds, the isolation and study of each compound individually is very costly and inefficient. Considering that, a huge number of methods have been developed to evaluate the antioxidant capacity of plant extracts *in vitro*. The ABTS, FRAP or DPPH are among the methods most commonly used to evaluate the antioxidant capacity of brassica extracts. They could be used as a tool in breeding programs. Besides, due to the observed correlation among these methods it could be possible just to use one of them to determine the antioxidant capacity of a crop.

It is well known that carotenoids and phenolic compounds confer antioxidant capacity to the organism. In the particular case of the brassica crops, the antioxidant capacity has been related to their composition in phenolic compounds. These compounds are one of the major classes of secondary metabolites present in brassicas. They are involved in different physiological functions such as growth, reproduction, protection against pathogens and herbivores or UV radiation. Within the group of phenolic compounds we could find a major group of natural pigments, the anthocyanins. Due to their chemical structure and the structural diversity, these compounds act as antioxidants by donating hydrogen and electrons to free radicals or trapping them into their aromatic structure. Carotenoids are compounds very diverse that includes more than 600 natural pigments. Some of them are essential compounds in the human diet whereas others show positive effects against diseases.

Despite the generalized used of methods to determine the antioxidant capacity, little it is known about their mechanism and the genetics behind the regulations of these compounds. For this reason, one of the objectives of this thesis is to carry out a QTL analysis to study the genetic control of these quantitative traits. This study will allow the future identification of candidate genes responsible of the variation of the studied traits.

Glucosinolates (GSLs) do not have antioxidant activity '*per se*', but could be considered antioxidant compounds because they can enhance the enzymatic antioxidant systems within the cell. These compounds are the most important group of secondary metabolites in the *Brassica* genus due to their uniqueness since these compounds are present in the nature exclusively in the species of the order Brassicales where is included the *Brassica* genus. More than 200 GSLs have been identified on basis of modifications in the lateral chain. Based on the precursor amino acid, GSLs are classified into three major groups namely aliphatic, indolic, and aromatic.

Variation in composition and concentration of GSLs depends on several factors among which are both, genetic and environmental factors. Furthermore, development stage and various post-harvest and processing factors also are involved. Each *Brassica* species, has a characteristic pattern of GSLs, usually 10 or 12 different GSLs appears within each crop. The majority of GSLs present in *B. oleracea* are aliphatic. Actually, it is known that the GSLs hydrolysis products are the responsible of plant defense system and are also responsible for the beneficial health properties attributed to these compounds. Several studies had determined that the mechanisms of action of the compounds derived from GSLs on human health include modulation of enzymes involved in carcinogenesis processes, protection against oxidative stress and inhibition of tumor development. Due to these properties, significant efforts have been made to locate the major genes responsible for the biosynthesis of GSLs in different species of the genus *Brassica.* This knowledge will provide future possibilities to manipulate and increase the GSLs content with beneficial properties for human health into the crops.

To date, some of the major genes related with the aliphatic GSLs biosynthesis have been identified. However, other genes responsible for different stages of the biosynthesis pathway of aliphatic, aromatic and indolic GSLs remain to be identified. There are different molecular techniques, like the QTLs analysis, to identify genes involved in the biosynthesis pathway. Although *B. oleracea* is one of the most

important species from an economic point of view of its genus, to date this is the first study of QTL mapping for the content GSLs in this species.

In many horticultural crops, such as those found in *B. oleracea*, breeding programs have mainly served to aspects such as productivity, disease resistance, product uniformity and external quality. However, in recent years brassica breeding programs have been incorporated the content in bioactive compounds within their objectives due to interest in healthy products. Thus, modification of the content of GSLs becomes a new target in the improvement of this species. Knowledge of the genetic bases of their synthesis and regulation provides the ability to manipulate GSLs profiles. To date, the modification of the content of GSLs had been made by introgression from wild germplasm or by genetic transformation. Therefore, in this thesis, mass selection for content in different individual GSLs was conducted in leaves. This method has been practiced by farmers over the centuries to enhance desirable characteristics in different crops and it is still used today in breeding programs when the speed of the process is essential, and the character present high heritability. Furthermore, in the case of *B. oleracea* crops this method is easier to apply due to the character of the cross pollinated species and the difficulty for manual crosses.

With this background, the main objective of this thesis is to extend the knowledge about the mechanisms or genes involved in the biosynthesis and accumulation of different beneficial compounds to human health presents in *B. oleracea* such as compounds with antioxidant activity and GSLs. The relationship between the different methods to measure antioxidant activity and its relation to the content of phenolic compounds, anthocyanins and carotenoids will be studied. QTLs related to antioxidant activity methods, metabolites with antioxidant activity and GSLs content will be searched as a prerequisite to the identification of potential candidate genes. Furthermore, the effect to the divergent selections in the content of the three major GSLs in kale leaves will be determined. Parallel the side effect of divergent selections in seeds and flower buds, and the side effect on the content of other GSLs in leaves, flower buds and seeds will also be study.

In this thesis, a comparison of three antioxidant activity methods (FRAP, ABTS and DPPH) was conducted in a doubled haploid (DH) mapping population in two different plant organs (leaves and flower buds). Results suggest that it is necessary to

use more than on method in order to estimate the antioxidant activity of a variety. This is due to the fact that these methods present low significant correlations between them because the material employed in this thesis, meaning the DH lines, is genetically related.

Different metabolites that may be causing the antioxidant activity, as anthocyanins, total phenolics compounds and carotenoids were analyzed. In this case, correlations between different measurement methods and the content of total phenolics and carotenoids compounds were found, confirming that these compounds are partly responsible for the antioxidant activity of brassica crops.

A total of 19 QTLs, 9 in leaves and 10 in leaves in flower buds were found. Common genomic regions for methods to measure antioxidant activity and content of metabolites responsible for it were detected. To date, there are no published works on brassica crops oriented to evaluate and compare different methods of measuring antioxidant activity and to identify related QTLs.

A genetic study of GSLs was conducted in three different organs of plants, leaves, seeds and buds on a DH population of *B. oleracea*. Results obtained in this study show that there are more concentration of GSLs in the reproductive organs than in the vegetative ones. In this way, the highest content of GSLs was found in the seeds, followed by the flower buds, and finally, in the leaves. Regarding the searching for QTLs and candidate genes involved in the GSLs biosynthesis pathway, a total of 20 QTLs consensus were found. Two of these consensus QTLs were seed specific, two were flower buds specific and one was leave specific.

Detection of organ-specific QTLs in the study of antioxidants and GSLs, assumes that part of the regulation of synthesis routes is dependent on the organ. Therefore, it could be possible to obtain enriched varieties in a specific compound in a specific organ in the plant. Three genomic regions controlling variability and content of compounds with antioxidant activity and GSLs were found in the chromosomes 3, 5 and 7. In the three genomic regions, alleles increasing the traits are given by the same parent.

In both works of QTLs, and thanks to the genetic relatedness of the species under study, *B. oleracea* and the model plant *Arabidopsis thaliana*, a synteny study was

undertaken for both species in order to identify the homologous genes in *B. oleracea* which control GSLs content, methods to measure antioxidant activity and metabolites with antioxidant activity. In fact, genes related to the synthesis pathway of phenolic compounds and GSLs were located by '*in silico'* mapping in the confidence intervals of QTLs detected all along this thesis. Specifically, in the study of antioxidants, several key genes of the route of phenylpropanoids (related to the synthesis of phenolic compounds) as HCT and C3'H were located in the chromosome 3. This region is shown as an area of specific importance because QTLs related to the three methods employed (FRAP, ABTS, DPPH) were detected on it. In the other hand, main loci controlling major part of variability for aliphatic GSLs GSL-OH, GSL-PRO y GSL-ALK were identified. In the case of indolic and aromatic GSLs, genes CYP79B2, CYP81F2, CYP79B3 y ATR1 were located.

All candidate genes can be studied and corroborated in brief because one of the parents of the mapping population employed for the QTLs studies, parent TO1000DH3, has been used to sequence *B. oleracea.* Sequences (although still incomplete) have been recently published. This publication will allow corroborating our results in a near future. Besides, it will facilitate searching in a faster and direct way other genes implied in the biosynthesis route of the different metabolites under study.

In addition to the identification of QTLs and candidate genes, the determination of possible epistatic relationships among identified loci is of great importance. These could greatly influence variability and content of metabolites under study. In the genetic study of GSLs, a high number of epistatic interactions were detected, being some of them common to the three studied organs. Among all the interactions, the locus GSL-ALK outstands because of its role in determining variation of aliphatic GSLs. This locus is in the center of a network of epistatic interactions, where up to 12 QTLs are involved.

To verify whether masal selection can be an effective method to increase and/or decrease the content of a particular GSL, and to obtain material with the content of a particular GSL modified, three divergent selections performed in a local population of kale (*Brassica oleracea* var. *acephala*) were evaluated. Selections programs were made for each one of the main GSLs present in the leaves: two aliphatic GSLs sinigrin (SIN) and glucoiberin (GIB) and one indolic GSL glucobrassicin (GBS). Results show that it

is possible to quantitatively modify the concentration of the GSLs cited above in the same genetic background. This indicates that the concentration of GSLs is a highly heritable trait and that can be modified through conventional selection. The response adjusts to a model of linear regression and there are no signs that the genetic variability for this trait is exhausted on leaves in none of the directions. It may be possible to obtain new selection cycles successfully.

In addition, the effect of the modification of GSLs made in leaves on the composition and concentration of the same GSLs in flower buds and seeds was evaluated. A secondary effect of the selection on these organs was found. This indicates that there is a modification of the synthesis of GSLs in flower buds and seeds, or there is translocation of GSLs synthesized in leaves to these other organs. Besides, altering the concentration of particular GSLs has an effect on the rest of GSLs of the plant, mainly on those which are in the same synthesis route. Indirect effects caused by the selections for aliphatic GSLs (SIN y GIB) on the rest of GSLs of the plant suggested that different alleles of the locus GSL-ALK could be responsible for the variation of the concentration across selection cycles. Besides, results support the hypothesis of the locus GSL-ALK controlling indirectly the variability of the indolic GSL named GBS, indicating a relationship between both biosynthetic routes.

Comparing both independent GSLs works of this thesis (search of genes and modification in the composition) we can conclude that, the locus GSL-ALK is responsible of great part of the observed variability of aliphatic GSL. Because of that, it seems obvious that this locus should be taken into account when planning breeding programs to modify GSLs content. Selecting this locus could have indirect effects on other genes of the same biosynthetic route.

In conclusion, data provided by this work may be very useful in deepen the knowledge about the synthesis and regulation of health-promoting compounds in *B. oleracea* crops.

ÍNDICE

ÍNDICE

CAPÍTULO IV: IDENTIFICATION OF METABOLIC QTLS AND CANDIDATE GENES FOR GLUCOSINOLATE SYNTHESIS IN *BRASSICA OLERACEA* LEAVES, SEEDS AND FLOWER BUDS

ÍNDICE DE ABREVIATURAS Y SÍMBOLOS

4CL: 4-cumarato coenzima A ligasa.

AA: actividad antioxidante.

ABTS: ácido 2,2'azino-bis (3-etilbenzotiazolina) 6-sulfónico.

ALA: alanina.

ALY: glucoalisina (5-metilsulfinilpentil).

cM: centimorgan.

C4H: cinamato 4-hidroxilasa.

CHS: chalconas.

CoA: coenzima A.

DH: dobles haploides.

DPPH: 2, 2-difenil-1-picril-hidrazilo.

DW: dry weight (peso seco).

ET: electron transfer (transferencia de electrones).

FAO: Food and Agricultural Organization of the United Nations (Organización de las Naciones Unidas para la Alimentación y la Agricultura).

FRAP: ferric reducing antioxidant power (capacidad de reducción del hierro férrico).

GBS: glucobrasicina (3-indolilmetil).

GER: glucoerucina (4-metiltiobutil).

GIB: glucoiberina (3-metilsulfinilpropil).

GIV: glucoiberverina (3-metiltiopropil).

GNA: gluconapina (3-butenil).

GNT: gluconasturtina (2-feniletil).

GRA: glucorrafanina (4-metilsulfinilbutil).

GSL: glucosinolato.

ILEU: isoleucina.

LEU: leucina.

MAM: enzima metiltioalquil sintasa.

MET: metionina.

NeoGBS: neoglucobrasicina (1-metoxi-3-indolilmetil).

OHGBS: hidroxiglucobrasicina (4-hidroxi-3-indolilmetil).

OMS: Organización Mundial de la Salud.

QTL: quantitative trait locus (locus de un carácter cuantitativo).

P450: citocromo 450.

PAL: phenylalanine ammonia-lyase (fenilalanina amonio liasa).

PHE: phenilalanin (fenilalanina).

PRO: progoitrina (2-hydroxy-3-butenyl).

RFLPs: restriction fragment length polymorphism (polimorfismos de longitud de fragmentos de restricción).

RNAi: RNA de interferencia.

ROS: reactive oxygene species (especies reactivas del oxígeno).

S-GT: S-glucosiltransferasa.

SFN: sulforrafano.

SIN: sinigrina (2-propenil).

SS: stilbene (estilbeno).

SSRs: simple sequences repeat (microsatélites).

TE: transferencia de electrones.

TPTZ: 2,4,6-tripitidilestriacina.

TROLOX: 6-hidroxi-2,5,7,8-tetrametilcroman-2- ácido carboxílico.

TRP: triptófano.

TYR: tyrosine (tirosina).

UHPLC: ultra high performance liquid chromatography (cromatografía líquida de ultra alta eficacia).

USDA: United States Department of Agriculture.

UV: ultravioleta.

VAL: valina.

CAPÍTULO I

INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Descripción del género *Brassica*

El género *Brassica* pertenece a la familia *Brassicaceae* (tribu *Brassiceae*, subtribu *Brassicinae*), integrada por 350 géneros y en torno a 3.500 especies. Su distribución se circunscribe fundamentalmente a las regiones templadas del hemisferio norte (Nuez et al., 1999), si bien es una familia cosmopolita debido a su capacidad de adaptación a un amplio rango de condiciones climáticas, ya que se consideran plantas moderadamente resistentes a las heladas y altamente resistentes a las sequías (Prakash e Hinata., 1980; Nieto, 1996; Rosa, 1999).

Taxonómicamente el género *Brassica* comprende un número considerable de especies y cultivos con una gran diversidad de características biológicas, debido a los distintos procesos de selección natural, aparición de mutaciones, posibles introgresiones de material genético procedente de especies silvestres y a los diferentes usos que surgieron con los diferentes procesos de domesticación (Prakash e Hinata, 1980). Actualmente lo integran 37 especies (Gómez-Campo, 1980) (Figura 1.1); sin embargo, la importancia económica de este género se centra de manera casi exclusiva en seis. U (U, 1935) estableció las relaciones filogenéticas entre estas seis especies basadas en estudios citológicos y de relación entre sus genomas, a través de una figura conocida como el triángulo de U (Figura 1.2), en cuyos vértices se disponen las tres especies diploides, *Brassica nigra* (L.) Koch (n=8), *Brassica oleracea* L. (n=9) y *Brassica rapa* L. (n=10). Tanto *B. rapa* como *B. oleracea* se habrían originado a partir de un ancestro común de seis cromosomas, mientras que *B. nigra* habría evolucionado a partir de una ancestro diferente, estando más próximo a *Sinapis alba*, un tipo de mostaza. Los genomas de *B. rapa*, *B. nigra* y *B. oleracea* han sido denominados A, B y C, respectivamente. Las especies anfidiploides *Brassica juncea* (L.) Czern. (n=18), *Brassica napus* L. (n=19) y *Brassica carinata* A. Braun (n=17), se disponen en los laterales y sus genomas se denominan AB, AC y BC, respectivamente. La mayoría de las especies de brásicas diploides son autoincompatibles y se consideran predominantemente alógamas. Las especies anfidiploides son fundamentalmente autógamas, aunque con una tasa variable de alogamia parcial (Becker et al., 1999; Soengas et al., 2011).

Figura 1.1: Diversidad dentro del género *Brassica.*

Las especies *B. oleracea* y *B. rapa* contienen la mayoría de los cultivos agrícolas del género. La mayor variabilidad genética y fenotípica de *B. oleracea* se encuentra en Europa, mientras que Asia representa el principal ámbito de la diversificación de los cultivos de *B. rapa*. Los cultivos de mayor distribución mundial pertenecen a la especie *B. oleracea* e incluyen formas hortícolas y forrajeras, como la col rizada, repollo, brécol, coles de Bruselas y coliflor entre otros. *B. rapa* incluye formas hortícolas como el nabo, col china y pak-choi, junto con algunos cultivos forrajeros y oleaginosos y *B. napus* contiene principalmente variedades que se utilizan para la producción de aceites a partir de sus semillas, como la colza, aunque contiene otras hortícolas de hoja y forrajeras como el nabicol y el colinabo, respectivamente. Por último, el grupo de las mostazas está formado por *B. carinata*, *B. nigra* y *B. juncea*, las cuales se usan principalmente como condimento a partir de sus semillas, aunque de *B. juncea*, se utilizan también sus hojas y las pellas o cabezas para el consumo hortícola, principalmente en los países asiáticos.

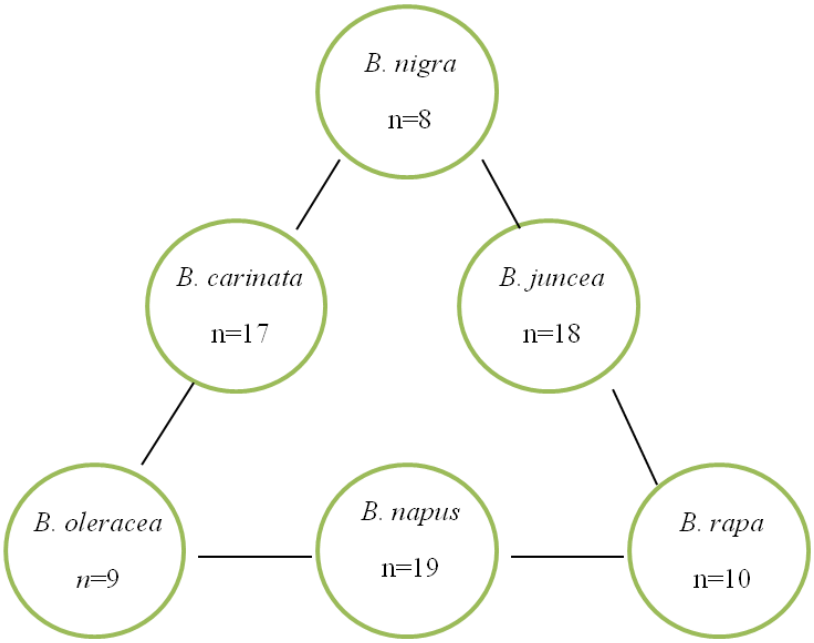


Figura 1.2: Triángulo de U (1935).

1.2. Origen y evolución de *Brassica oleracea*

Los inicios del cultivo de brásicas datan de finales del neolítico. El origen del cultivo de *B. oleracea* tuvo lugar en las costas del noroeste europeo. De Candolle (1886, citado por Prakash e Hinata., 1980) afirma que fueron los celtas los que domesticaron la especie, aunque también sugiere que estos pudieron encontrársela ya cultivada por pueblos autóctonos cuando invadieron esta región europea entre los siglo VI-VIII a. C. Algunas teorías proponen un origen polifilético por adaptación de diferentes especies mediterráneas (Snogerup, 1980; Mithen et al., 1987), aunque estudios posteriores no encontraron evidencias moleculares que sugieran líneas paralelas de origen para los diferentes cultivos de *B. oleracea* (Hosaka et al., 1990). Actualmente se piensa que procesos de hibridación e introgresión de *B. oleracea* con especies silvestres (n=9) pudieron contribuir al desarrollo de las distintas variedades hortícolas aumentando su variabilidad y adaptabilidad, pero siempre otorgando a *B. oleracea* el papel principal en el desarrollo de las distintas formas de cultivo (Tabla 1.1) (Gómez-Campo, 1999).

Los usos de las brásicas son tan variables como sus formas y productos, existiendo aplicaciones en la alimentación humana, la alimentación animal, la industria y la ornamentación. *B. oleracea* limita su aprovechamiento a los tejidos epicotilares vegetativos (berzas, repollo, colirrábano…) y fructíferos (brécol y coliflor) (Figura 1.3). La mayoría de los cultivos de esta especie tienen un uso hortícola y sus partes verdes se consumen en crudo o cocinados. Muestra de su importante valor culinario es que las brásicas son el ingrediente fundamental de platos tradicionales de algunos países como son el cocido en España, el caldo verde en Portugal o el chucrut en Alemania, entre otros.

Figura 1.3: Cultivos de *Brassica oleracea*: A) berza, B) repollo, C) coliflor, D) colirrábano, E) brécol y F) coles de Bruselas.

B. oleracea también está presente en la alimentación animal pudiendo utilizarse como suplemento en forma de forraje. Esta especie también es fácil verla como planta ornamental de exterior, ya que es habitual encontrarla en zonas verdes públicas decorando parques, parterres y bancales en los meses de invierno.

Tabla 1.1: Variedades de importancia económica dentro de la especie *Brassica oleracea.*

Especie	Grupo	Nombre común del cultivo	Parte consumida
Brassica oleracea	*acephala*	Berza	Hojas e inflorescencias
	capitata capitata	Repollo	Hojas
	capitata sabauda	Repollo de hojas rizadas	Hojas
	costata	Asa de cántaro	Hojas
	gemmifera	Coles de Bruselas	Brotes axilares
	botrytis botrytis	Coliflor	Inflorescencias
	botrytis italica	Brécol	Inflorescencias
	gongylodes	Colirrábano	Hipocotilo
	alboglabra	Col china	Hojas

1.3. Calidad nutricional de *Brassica oleracea*

La relación existente entre los hábitos alimenticios y el desarrollo de ciertas dolencias ha impulsado el incremento de una conciencia social hacia la mejora de la alimentación, así como de las instituciones gubernamentales y organismos internacionales (OMS, FAO, USDA) para desarrollar campañas dirigidas a estimular el consumo de alimentos de origen vegetal y llevar una alimentación más saludable como medida preventiva frente al desarrollo de enfermedades. Numerosos estudios *in vitro* en animales modelo, así como ensayos clínicos y estudios epidemiológicos, indican que el consumo de frutas y hortalizas unido a una dieta equilibrada, se relaciona con un efecto protector frente al riesgo de padecer enfermedades cardiovasculares, neurodegenerativas y diversos tipos de

cáncer (Verhoeven et al., 1996; van Poppel et al., 1999; Murillo y Mehta, 2001; Kristal y Lampe, 2002), incidiendo favorablemente en la mejora de la calidad de vida de las personas (Arts y Hollman, 2005; Kim y Park, 2009).

En este punto es donde los vegetales en general, y las brásicas en particular, juegan un papel importante en la salud debido a sus propiedades beneficiosas (Ayaz et al., 2006; Nilsson et al., 2006). Desde un punto de vista nutricional, las brásicas comparten las características generales del resto de productos hortícolas, como es el bajo contenido en grasa y alto contenido en fibra y minerales (Hernández, 1989), particularmente en potasio, cobre, magnesio, manganeso, hierro, zinc y calcio (Kopsell et al., 2004; Ayaz et al., 2006), siendo este último muy abundante en berzas y brécol (Bicudo et al., 1990; Farnham et al., 2000; Kopsell et al., 2004). Además, presentan importantes niveles de vitaminas, destacando el contenido en vitamina C, responsable de que estos vegetales hayan sido utilizados históricamente en medicina para combatir el escorbuto (Rosa, 1999; Ordás, 2000).

Las brásicas, además, contribuyen a prevenir ciertas enfermedades debido a su composición rica en ciertos metabolitos secundarios. Los productos del metabolismo secundario de las plantas fueron clasificados en un principio como simples productos de desecho (Rosa, 1999). Sin embargo, se han descubierto numerosas y complejas funciones biológicas asociadas a los mismos. Estos compuestos actúan como sustancias de defensa frente a plagas y enfermedades y también son los responsables de ciertas propiedades organolépticas, como el sabor amargo característico de algunos cultivos (Jones y Sanders, 2002; Schonhof et al., 2004). En este grupo se encuentran diversas sustancias nutritivas que intervienen en el metabolismo secundario de las plantas, que sin presentar una función nutricional clásicamente definida y sin considerarse esenciales para la salud humana, tienen un impacto significativo en esta (Hooper y Cassidy, 2006). En los últimos años los compuestos bioactivos han sido objeto de una importante labor de investigación acerca de sus propiedades, absorción, metabolismo y actividad biológica (Manach et al., 2004), aunque todavía es necesario llevar a cabo estudios exhaustivos para comprender los mecanismos de síntesis así como de acción sobre la salud humana (Jeffery y Keck, 2008; Jeffery y Araya, 2009).

Entre los compuestos bioactivos presentes en los cultivos de brásicas destacan de modo especial los glucosinolatos (GSLs) y diversos compuestos con actividad

antioxidante entre los que se incluyen los carotenoides y compuestos fenólicos (flavonoides y antocianinas), los cuales están adquiriendo un interés creciente por sus múltiples efectos beneficiosos. En los últimos años se ha demostrado que muchos de ellos tienen efectos protectores contra el daño oxidativo asociado con una reducción de padecer enfermedades crónicas (Okarter y Liu, 2010), así como un efecto citotóxico y apoptótico en células dañadas, como las células cancerígenas. También reducen el riesgo de padecer enfermedades degenerativas o cardiovasculares (Hooper y Cassidy, 2006; Fahey y Kensler, 2007a; Forte et al., 2008; Van Horn et al., 2008; Virgili y Marino, 2008). El efecto que sobre la salud humana y la protección de las plantas tienen los metabolitos con actividad antioxidante y los GSLs en las brásicas, hacen que estos caracteres sean interesantes a la hora de incluirlos en programas de mejora con el fin de obtener cultivos enriquecidos en estos compuestos.

1.3.1. Las brásicas como fuente de antioxidantes

Las diferentes especies del género *Brassica* presentan una importancia substancial en la dieta humana debido a que son una fuente importante de nutrientes y compuestos antioxidantes (Podsedek, 2007). Los antioxidantes fueron comunes en la industria química y de la alimentación durante los siglos XIX y XX. Sin embargo, no fue hasta los años 60 cuando algunos estudios revelaron la importancia de los antioxidantes en la salud, con publicaciones acerca del efecto de los flavonoides, el ácido ascórbico y el estrés oxidativo.

El estrés oxidativo surge en sistemas biológicos después de una prolongada exposición a oxidantes, o a una disminución de la capacidad antioxidante del sistema o a ambas. En el proceso de respiración celular y en la fotosíntesis se producen radicales libres de oxígeno y nitrógeno (ROS/RNS), los cuales pueden causar daño oxidativo en el ADN, proteínas y en los lípidos de membrana. Estos radicales libres están fuertemente implicados en el envejecimiento prematuro y en la patología de enfermedades cerebrales, cardiacas, arterosclerosis y cáncer, entre otras. La exposición a los ROS y RNS ha hecho que los organismos desarrollen una serie de mecanismos de defensa que incluyen sistemas enzimáticos y no enzimáticos. Entre los sistemas de defensa no enzimáticos se

incluyen diversos compuestos bioactivos con actividad antioxidante. La ingesta de estos compuestos bioactivos presentes en los vegetales protege las células contra el daño oxidativo, induce la detoxificación de enzimas y estimula el sistema inmune; por tanto, pueden prevenir enfermedades crónicas como el cáncer, la diabetes y enfermedades cardiovasculares (Murillo y Mehta, 2001; Kristal y Lampe, 2002; Higdon et al., 2007; Jahangir et al., 2009; Kapusta-Duch et al., 2012). Los sistemas de defensa antioxidante están además relacionados con la resistencia de la planta a distintos estreses.

Las brásicas son una buena fuente de estos antioxidantes naturales. Su actividad antioxidante es alta comparada con otras hortalizas; de hecho, el brécol y las coles destacan entre los cultivos que presentan una elevada actividad antioxidante superando a la espinaca, la zanahoria, la patata, el pimiento verde, la remolacha, el ruibarbo y la judía (Ou et al., 2002; Zhou y Yu, 2006). El beneficio en la salud humana asociado al consumo de brásicas podría estar explicado en parte, por su elevada composición en antioxidantes, por lo que consecuentemente estos cultivos han sido el foco de una intensa búsqueda basada en el contenido de metabolitos secundarios relacionados con esta función (Traka y Mithen, 2009; Verkerk et al., 2009). Entre estos compuestos bioactivos con actividad antioxidante se encuentran las vitaminas, especialmente las vitaminas A, B6, β-caroteno, luteína, zeaxantina y vitamina K (Dekker et al., 2000; Vallejo et al., 2004), folato, azúcares solubles (Pedroche et al., 2004), lignina y compuestos fenólicos como los antocianos (Heimler et al., 2006). Debido a la complejidad de la composición en antioxidantes de los extractos vegetales, la separación de cada compuesto y el estudio de su actividad individual son costosos y complicados. La actividad antioxidante total va a venir determinada a su vez, por interacciones sinérgicas o inhibitorias entre los diferentes compuestos así como por el modo de acción concreto de cada uno de ellos. En este sentido, durante los últimos años, se han desarrollado diferentes métodos químicos que permiten determinar la actividad antioxidante total de extractos vegetales *in vitro*.

1.3.1.1. Métodos para medir la actividad antioxidante

Existen varias aproximaciones para clasificar los distintos métodos utilizados para medir la actividad antioxidante. Huang et al. (2005) proponen una clasificación que describe los métodos según sus mecanismos de reacción, de transferencia de átomos de hidrógeno

(TAH) y de transferencia de electrones (TE), los cuales pueden ocurrir de forma paralela. Ambos métodos dan como resultado la estabilización del radical libre (Huang et al., 2005). En este manuscrito nos centraremos en los métodos basados en la transferencia de electrones (TE), que son los que se usan de forma más común con extractos de brásicas así como con otras frutas y verduras. Estos métodos determinan la actividad de un antioxidante para transferir un electrón y reducir un compuesto. La reactividad relativa de un antioxidante en un método TE está basada fundamentalmente en la desprotonación y, por lo tanto, es gobernada por el potencial de ionización. Entre los métodos TE más comúnmente usados se encuentran el método FRAP (ferric reducing/antioxidant power o actividad para reducir el hierro férrico), ABTS (2,2'-azino-bis (3-etilbenzoatiazolina-6-ácido sulfónico)) y DPPH (2,2-difenil-1-picril hidrazilo).

1.3.1.1.1. ***Método FRAP (ferric reducing/antioxidant power o capacidad para reducir el hierro férrico)***

El método FRAP fue desarrollado originalmente por Benzie y Strain (1996) para medir el poder reductor del plasma sanguíneo. Posteriormente, se adaptó para medir la actividad antioxidante de productos fitoterapéuticos y nutracéuticos (Benzie y Szeto, 1999). En este método se determina la actividad antioxidante de forma indirecta. Se basa en el poder que tiene una sustancia antioxidante para reducir el Fe^{3+} a Fe^{2+} que es menos antioxidante. A bajo pH se detecta un color azul intenso que puede ser monitorizado a 593nm. Así, cuanto más antioxidante es la sustancia objeto de estudio, mayor es la reducción y mayor la concentración de Fe^{2+}y más alta la señal de absorbancia. Debido a que el potencial redox del Fe^{3+} TPTZ (2,4,6-tripitidilestriacina) es comparable con el del ABTS+ se pueden analizar compuestos similares con ambos métodos aunque las condiciones de la reacción sean distintas.

1.3.1.1.2. *Método ABTS (2,2'-azino-bis (3-etilbenzoatiazolina-6-ácido sulfónico))*

Este método fue descrito inicialmente por Miller et al. (1993) y se basa en la capacidad de un antioxidante para estabilizar el radical catión coloreado ABTS+. Este compuesto es de color verde-azulado, estable y con un espectro de absorción en el UV-visible.
Entre las ventajas de este método cabe citar que se puede utilizar en un amplio rango de pHs y fuerza iónica, es soluble tanto en medio acuoso como orgánico y permite la evaluación de antioxidantes hidrofílicos y lipofílicos. Entre las desventajas se encuentra el punto final de medida, ya que debe fijarse de manera arbitraria o de forma experimental debido a que la cinética de reacción con algunos antioxidantes suele ser lenta (Figura 1.4).

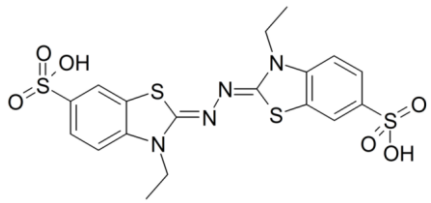


Figura 1.4: Estructura química del radical ABTS.

1.3.1.1.3. *Método DPPH (2,2-difenil-1-picril hidrazilo)*

Este método fue propuesto originalmente por Brand-Williams et al. (1995). Se fundamenta en la medición de la capacidad de un antioxidante para estabilizar el radical DPPH (Figura 1.5). Este compuesto es uno de los pocos radicales orgánicos estable que presenta una coloración violeta. Entre las ventajas de este método se encuentran su simplicidad y el bajo requerimiento instrumental; la principal desventaja radica en la dificultad de interpretar los resultados cuando se trabaja con sustancias cuyo espectro de absorción se solapa con el del radical (a 517nm).

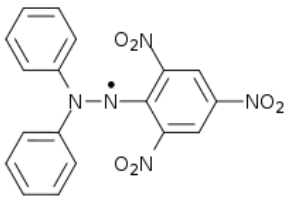


Figura 1.5: Estructura química del radical DPPH.

Normalmente, para medir la actividad antioxidante de un extracto se aplican varios métodos a la vez. En general, las correlaciones encontradas entre estos tres métodos en extractos de brásicas son elevadas, por lo que en teoría sería posible aplicar uno solo de ellos. La actividad antioxidante de los extractos de brásicas se relaciona normalmente con su contenido en compuestos fenólicos (Podsedek, 2007). Sin embargo, otro tipo de compuestos antioxidantes presentes en las brásicas como las antocianinas y los carotenoides también pueden estar relacionados con la misma.

1.3.1.2. Compuestos con actividad antioxidante

Normalmente, la actividad antioxidante de las brásicas se relaciona en mayor medida con su composición en compuestos fenólicos. Bajo este nombre se incluyen un gran número de compuestos (más de 8.000) distribuidos por todo el reino vegetal y caracterizados por presentar grupos hidroxilo unidos a uno o varios anillos aromáticos, lo que les confiere capacidad de captar radicales libres (Wang y Jiao, 2000). Los compuestos fenólicos varían desde un peso molecular bajo a moléculas complejas derivadas de taninos y polifenoles (Crozier et al., 2006; Pereira et al., 2009). Atendiendo a su estructura básica, los compuestos fenólicos se pueden dividir en dos grupos: flavonoides y no flavonoides (Robards et al., 1999). Los flavonoides incluyen diferentes tipos de compuestos como flavonoles, antocianos, flavan-3-oles, proantocianidinas, flavanonas, flavonas, isoflavonas y chalconas. Dentro de los compuestos no flavonoides se incluyen los ácidos hidroxicinámicos, ácidos hidroxibenzoicos, taninos hidrolizables y estilbenos (Robards et al., 1999).

Los compuestos fenólicos constituyen una de las principales clases de metabolitos secundarios presentes en los cultivos de brásicas, donde desempeñan diversas funciones fisiológicas (Apak et al., 2007; Cartea et al., 2011; Kaulmann et al., 2014). Entre otras, intervienen en el crecimiento y reproducción de las plantas, la protegen frente a factores ambientales adversos y contribuyen en procesos defensivos frente al ataque de patógenos, predadores o radiación ultravioleta (Duthie y Crozier, 2000; Havsteen, 2002). Las propiedades antioxidantes de estos compuestos están relacionadas con la inhibición de algunos enzimas y con la prevención de la formación de radicales libres inducida por metales como el hierro y el cobre (Scalbert et al., 2005; Dai y Mumper, 2010). Estas

propiedades hacen que los compuestos fenólicos se encuentren relacionados de una manera u otra con la salud humana (Zhang y Hamauzu, 2003). Se consideran como unos muy buenos antioxidantes con mayor eficacia que las vitaminas C, E y los carotenoides (Podsedek, 2007).

Los antocianos constituyen el grupo más importante de pigmentos naturales dentro del grupo de compuestos fenólicos (Harborne y Williams, 2000). Son responsables de los colores azul, violeta, rojo y naranja en la mayor parte de las plantas. Presentan moléculas de muchísimo interés debido a su impacto en las características sensoriales de muchas frutas y verduras así como propiedades beneficiosas para la salud debido a sus actividades biológicas (He et al., 2010; Yang et al., 2011). Desde el punto de vista estructural, los antocianos son flavonoides con el anillo C insaturado. Las distintas sustituciones en las posiciones 3', 4' y 5' del anillo B dan lugar a las diferentes antocianidinas (agliconas). Las agliconas se suelen encontrar unidas a uno o más azúcares, normalmente glucosa o ramnosa, formando así las antocianinas o antocianos (Clifford, 2000; De Pascual y Sánchez-Ballesta, 2008). Su estructura química es la adecuada para actuar como antioxidantes, donando hidrógenos y electrones a los radicales libres o bien atrapándolos en su estructura aromática (Miller y RiceEvans, 1997; Wang et al., 1997). La diversidad estructural contribuye favorablemente a la existencia natural de unos 300 antocianos con diferentes sustituciones glucosídicas (Harborne y Williams, 2000). Los principales antocianos identificados en crucíferas (como la lombarda o el brécol) son la cianidina 3-*O*-(sinapoil) (feruloil) diglucósido-5-*O*-glucósido y la cianidina 3-*O*-(sinapoil) (sinapoil) diglucósido-5-*O*-glucósido, con diferencias cuantitativas según cultivares (Wu y Prior, 2005; Moreno et al., 2010).

Los carotenoides son un grupo muy diverso en el cual se incluyen más de 600 pigmentos naturales (amarillos, naranjas y rojos) que se acumulan en los plástidos de las hojas, flores y frutos (Paiva y Russell, 1999). Algunos de ellos son nutrientes esenciales en la dieta humana, mientras que otros muestran efectos positivos frente a diferentes enfermedades. Algunos carotenoides son precursores de la vitamina A. Los carotenos más abundantes en los cultivos de brásicas son el β-caroteno (Figura 1.6) y la luteína, si bien se han identificado 16 carotenoides diferentes en extractos de brásicas (Wills y Rangga, 1996; Podsedek, 2007).

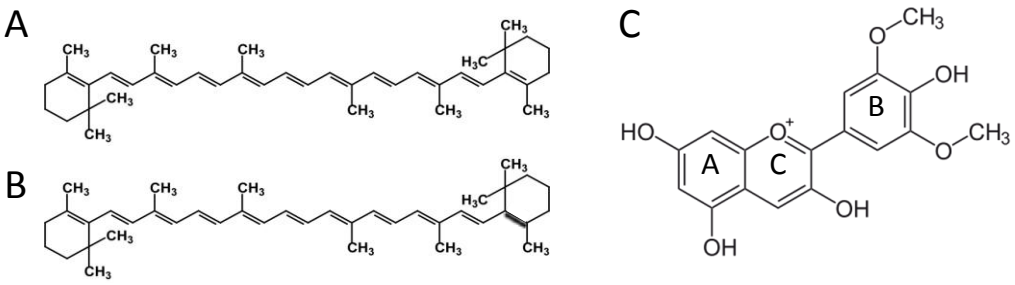


Figura 1.6: Estructura molecular de α-β caroteno (A y B) y de una antocianina (C).

Dentro de la especie *B. oleracea*, la berza es uno de los cultivos con mayor contenido en carotenoides (sobre 10 mg/100g parte comestible), siendo las coles de Bruselas un cultivo con valores intermedios (6,1mg/100g) y el brécol (1,6 mg/100g) o el repollo blanco (0,26 mg/100g) los cultivos con menor contenido en carotenoides totales (Muller, 1997).

1.3.1.3. Estudios genéticos de la actividad antioxidante en la actualidad

Los métodos para evaluar la actividad antioxidante se usan de forma extensiva con el fin de conocer las propiedades de distintos alimentos, debido a que son relativamente fáciles de manejar, baratos y bastante rápidos. Estos métodos de medida de compuestos con actividad antioxidante podrían ser además empleados como herramientas dentro de programas de mejora con el fin de obtener un material con mayor actividad antioxidante. A pesar de su uso cada vez más generalizado, actualmente se desconoce la genética básica de estos compuestos. Existen trabajos recientes en diversos cultivos no pertenecientes al género *Brassica* como el de Jin et al. (2009) en arroz, el de Dobson et al. (2012) en frambuesa y el trabajo de Hayashi et al. (2012) en lechuga. En estos trabajos se identificaron QTLs (quantitative trait locus) relacionados en la actividad antioxidante de compuestos fenólicos totales, antocianinas y carotenoides a partir de los extractos hidrofílicos de estos cultivos.

En el género *Brassica* existen diversos trabajos que relacionan la actividad antioxidante de diferentes cultivos con el contenido en un grupo específico de compuestos

como el kaempferol (Jung et al., 2009), la isoramnetina (Yokozawa et al., 2002) o la quercetina (Koh et al., 2009), entre otros. No obstante, sería necesario un análisis más exhaustivo para poder determinar con mayor certeza, los genes responsables de esta actividad. En las últimas décadas se ha generalizado el empleo del análisis de QTLs para analizar caracteres cuantitativos y medir el grado de asociación de marcadores genéticos con la variación fenotípica. Además, la identificación de QTLs es el primer paso para poder encontrar los genes responsables de la variación fenotípica de un carácter.

Generalmente se considera que los compuestos fenólicos son los principales compuestos con actividad antioxidante en los cultivos de brásicas. Su actividad antioxidante está relacionada con la neutralización directa de ROS y RNS, además de con la activación de ciertos sistemas enzimáticos. Sin embargo, también se considera que otro tipo de compuestos bioactivos, como los carotenos o los GSLs, son compuestos antioxidantes, no porque tengan actividad antioxidante '*per se*', sino porque contribuyen a potenciar los sistemas enzimáticos antioxidantes de la célula, induciendo la enzima tioredoxina-reductasa (uno de los antioxidantes más potentes presentes en los mamíferos) y poderosos agentes reguladores de reacciones redox celulares (Bao, 2005).

1.3.2. Los glucosinolatos

Los glucosinolatos (GSLs) son una clase de glucósidos azufrados químicamente estables, que se encuentran de manera exclusiva en la naturaleza en vegetales del orden Brassicales, en el que se incluye la familia *Brassicaceae* (Cartea y Velasco, 2008; Traka y Mithen, 2009). Son compuestos del metabolismo secundario de las plantas sintetizados a partir de aminoácidos directamente o previamente modificados (Rosa, 1999). Químicamente los GSLs están constituidos por un grupo β-D-tioglucósido unido a una oxima sulfonada y a una cadena lateral variable "R" derivada de un aminoácido (Figura 1.7) (Halkier y Du, 1997; Fahey et al., 2001; Grubb y Abel, 2006; Halkier y Gershenzon, 2006). Hasta la fecha, se han identificado más de 200 GSLs distintos, que difieren en las modificaciones sufridas en su cadena lateral "R" (Fahey et al., 2001; Clarke, 2010).

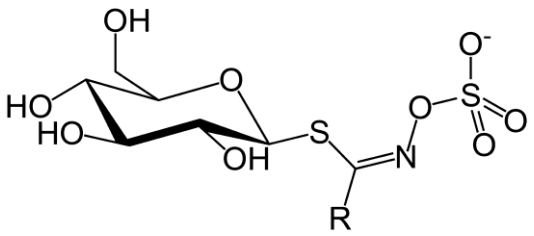
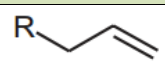
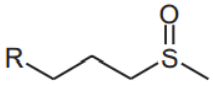
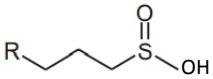
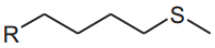
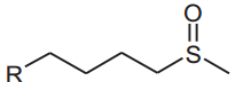
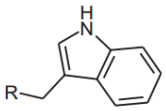
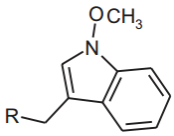
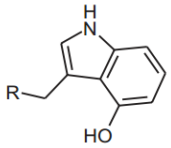
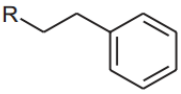


Figura 1.7: Estructura general de los glucosinolatos. R representa la cadena lateral variable a partir de los diferentes aminoácidos.

En función del aminoácido de procedencia, los GSLs se clasifican en alifáticos (derivados de la alanina, leucina, isoleucina, valina o metionina), indólicos (derivados del triptófano) y aromáticos (derivados de la fenilalanina o tirosina) (Figura 1.8) (Zukalova y Vasak, 2002; Vig et al., 2009). Los GSLs mayoritarios en *B. oleracea* son los alifáticos y, en menor medida, los indólicos (Tabla 1.2). Dentro de cada subespecie o tipo de brásicas, aparece el mismo patrón de GSLs. Normalmente existen entre 10 y 12 GSLs dentro de cada cultivo y de ellos tres o cuatro aparecen en concentraciones elevadas (Rosa, 1999). La variación de la composición y concentración de los GSLs depende de factores genéticos y ambientales. Además del genotipo, están involucrados el órgano de la planta analizado, la etapa de desarrollo, la temperatura, el tipo de suelo y el ambiente en el que se desarrolla, además de diversos factores post-cosecha y de procesado (Fenwick et al., 1983; Rosa, 1999; Kliebenstein et al., 2001a; Petersen et al., 2002; Rangkadilok et al., 2002; Brown et al., 2003; Farnham et al., 2004; Velasco et al., 2007; Cartea y Velasco, 2008; Wentzell y Kliebenstein, 2008).

Tabla 1.2: Clasificación y estructura de la cadena lateral de los glucosinolatos mayoritarios presentes en *Brassica oleracea.*

Tipo	Estructura de la cadena lateral	Nombre químico	Nombre común
Alifáticos			
		**2-Propenil**	Sinigrina
		**3-Metilsulfinilpropil**	Glucoiberina
		**2-Hidroxi-3-butenil**	Progoitrina
		**4- Metiltiobutil**	Glucoerucina
		**4-Metilsulfinilbutil**	Glucorrafanina
Indólicos			
		**3-Indolilmetil**	Glucobrasicina
		**1-Metoxi-3-indolilmetil**	Neoglucobrasicina
		**4-Hidroxi-3-indolilmetil**	4-Hidroxiglucobrasicina
Aromáticos			
		**2-Feniletil**	Gluconasturtina

1.3.2.1. Importancia biológica de los glucosinolatos

Los GSLs son producidos por las plantas como un mecanismo eficaz de defensa frente a herbívoros, nematodos, hongos y bacterias (Rosa, 1999; Vig et al., 2009). Aunque la relación planta - patógeno es bastante compleja, en algunos casos, se han observado efectos beneficiosos para algunos insectos especialistas que han conseguido adaptarse a la presencia de los GSLs en la planta (Giamoustaris y Mithen, 1995). Los GSLs se encuentran en todas las partes de la planta, aunque existen diferencias cualitativas y cuantitativas en función de la especie y el tejido, siendo la concentración de GSLs superior en los tejidos reproductivos (flores y semillas) (10-40 veces superior) que en tejidos vegetativos (Bennett et al., 2004). En la planta, la distribución de GSLs varía entre los distintos órganos: semillas >hojas jóvenes > tallo> sistema radicular > hojas maduras (Brown et al., 2003; Grubb y Abel, 2006; Velasco et al., 2007; Cartea y Velasco, 2008).

En realidad, no son los GSLs intactos sino los productos de hidrólisis resultantes de la degradación de los mismos los que suponen a la planta un sistema de defensa y son los responsables las propiedades beneficiosas para la salud atribuidas a estos compuestos (Van Poppel et al., 1999; Hecht, 2000). Diversos estudios *in vitro* e *in vivo* han determinado que los principales mecanismos de acción de los compuestos derivados de los GSLs en la salud humana incluyen la modulación de enzimas implicadas en procesos de carcinogénesis (iniciación, promoción y progresión) (Jeffery y Araya, 2009; Verkerk et al., 2009), la protección frente al estrés oxidativo (Cameron y Pauling, 1978; Ames et al., 1993) y la inhibición del desarrollo de tumores (inhibición de la proliferación celular e inducción de apoptosis) (Holst y Williamson, 2004; Clarke et al., 2008; Jeffery y Keck, 2008).

Además de todos estos efectos beneficiosos, es importante señalar que en animales se han descrito algunos efectos adversos relacionados con la ingesta de GSLs (Fahey et al., 2001; Anilakumar et al., 2006). Así, la oxazolidina-2-tiona, producto de hidrólisis del GSL progoitrina (2-hydroxy-3-butenyl GSL, PRO), posee la capacidad de bloquear la utilización y absorción del yodo, con lo que frena la actividad de la glándula tiroidea y puede llegar a provocar bocio en animales (Liu et al., 2012). Esto solo sucede cuando la concentración de PRO se encuentra en dosis muy elevadas, como la presente en la torta proteica que forma parte del pienso; sin embargo, hasta la fecha, no hay evidencias de

estos efectos tóxicos en humanos (Holst y Williamson, 2004; Cornblatt et al., 2007; Cartea y Velasco, 2008).

1.3.2.2. Biosíntesis y degradación de los glucosinolatos

La biosíntesis de los GSLs ocurre fundamentalmente en tres etapas. En la primera etapa se produce la elongación de la cadena lateral del aminoácido precursor, la segunda etapa consiste en la síntesis de la estructura central a partir del aminoácido modificado y en la tercera etapa se produce la modificación de la cadena lateral (Figura 1.8) (Fahey et al., 2001; Grubb y Abel, 2006). La elongación de la cadena lateral del aminoácido precursor requiere un total de cinco reacciones: transaminación inicial, condensación con acetil-CoA, isomerización, descarboxilación oxidativa y transaminación final. A continuación, se produce la conversión del aminoácido modificado a su correspondiente aldoxima, catalizado por las enzimas monooxigenasas de la familia CYP79 pertenecientes al complejo multienzimático citocromo P450 (CYP).

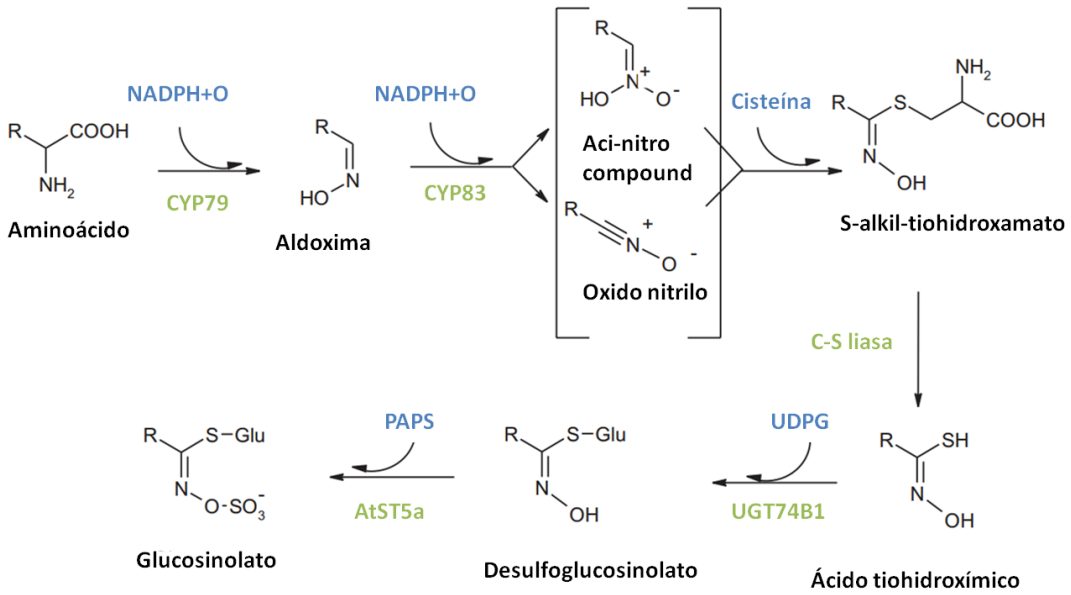


Figura 1.8: Esquema de las dos primeras etapas de la biosíntesis de los GSLs. En verde las enzimas que catalizan cada una de las etapas de la ruta de biosíntesis. Extraído y modificado de Redovnikovic et al., (2008).

Una vez formada la aldoxima se convierte en S-alquil-tiohidroximato mediante una reacción de oxidación catalizada por el citocromo CYP83 y por una reacción posterior de conjugación. Seguidamente la enzima C-S-liasa realiza la conversión del S-(alquil-tiohidroximato)-L-cisteína a ácido tiohidroxímico que posteriormente se convierte a desulfo-glucosinolato por la acción de glucosiltransferasas (UGT74B1). El paso final en la formación de estos compuestos es la sulfatación del desulfo-glucosinolato, reacción catalizada por la enzima PAPs soluble: desulfo-glucosinolato sulfotransferasa, formándose finalmente la estructura básica del GSL (aglicona). Por último, en la tercera etapa de la biosíntesis se producen las modificaciones en la cadena lateral que determinan la estructura final del GSL, dando lugar a los diversos GSLs presentes en la naturaleza (Grubb y Abel, 2006; Halkier y Gershenzon, 2006).

Los principales genes y factores de transcripción implicados en las tres etapas de la síntesis de GSLs han sido identificados y caracterizados en la planta modelo *Arabidopsis thaliana* (Halkier y Du, 1997; Halkier y Gershenzon, 2006). Gracias a que esta planta modelo pertenece al grupo de las crucíferas, la similitud genética es muy elevada con el género *Brassica* haciendo la identificación de genes más sencilla y certera. Basados en la homología con *A. thaliana*, se identificaron y clonaron inicialmente tres loci en *B. oleracea* (Li y Quiros, 2002; Li y Quiros, 2003; Gao et al., 2006) relacionados con la ruta de síntesis de los GSLs alifáticos. Dos de ellos, BoGSL-ELONG y BoGSL-PRO, son los responsables de la elongación de la cadena de GSLs alifáticos de 4 carbonos y 3 carbonos, respectivamente y son homólogos de los genes MAM-1 y MAM-2, respectivamente identificados en *A. thaliana*, mientras que el locus BoGSL-ALK es el responsable de la desaturación y producción de los GSLs alquenil y es homólogo del gen AOP2 de *A. thaliana*. Posteriormente, se mapearon otros genes en el genoma de *B. oleracea* como BoCS-liasa, BoGS-OH y BoCYP79F1 (Gao et al., 2007). Sin embargo, aún quedan muchos genes responsables de diferentes etapas de la ruta de biosíntesis de GSLs de los que no se tiene conocimiento, ni de su localización ni de su función. Hasta ahora, los trabajos realizados sobre las bases genéticas se han centrado mayoritariamente en el estudio de la ruta de los GSLs alifáticos. Aunque en los últimos años, ha despertado un creciente interés en la comunidad científica la ruta de síntesis y las propiedades de los GSLs indólicos, en la actualidad ambas siguen siendo prácticamente desconocidas (Chu et al., 2010; Pedras y Yaya, 2014; Vo et al., 2014).

Actualmente se sabe que la síntesis de todos los GSLs se produce en el citosol de las células vegetales (Mithen, 2001; Sonderby et al., 2010). La hidrólisis de los GSLs es llevada a cabo en las células vegetales por las tioglucosidasas, también conocidas con el nombre de mirosinasas (EC: 3.2.1.147), (Andreasson et al., 2000) encargadas de catalizar la hidrólisis de los GSLs en sus productos de degradación tras producirse una ruptura celular (Bones y Rossiter, 1996; Halkier, 1999). Estas enzimas se localizan en el citosol de células específicas llamadas células mirosinasas o idioblastos (Slupphaug et al., 1990), mientras que los GSLs se acumulan en las vacuolas de las "células S", de manera que mirosinasas y GSLs no están en contacto, pero sí en células adyacentes (Davies et al., 2000). Sólo cuando tiene lugar una rotura de tejidos reaccionan produciéndose una serie de productos resultantes entre los cuales destacan los isotiocianatos (ITCs), indoles, nitrilos, epi-nitrilos, oxazolidinas y tiocianatos (Figura 1.9) (Verkerk et al., 1997; Vig et al., 2009). Los productos de hidrólisis obtenidos dependerán de las condiciones de reacción existentes, ya que por ejemplo, el pH durante la hidrólisis influye tanto en la actividad mirosinasa como en la proporción de ITCs y nitrilos generados (Matusheski et al., 2001) (Figura 1.9).

Los isotiocianatos e indoles son los dos grandes grupos de descomposición autolítica de los GSLs. Ambos presentan actividades de protección contra muchos tipos de cáncer. Como la mayoría de las brásicas contienen una mezcla de GSLs, el efecto beneficioso de sus productos de degradación dependerá de la composición final. De todos los productos de degradación, el ITC más estudiado por su potente efecto quimio-protector es el sulforrafano (SFN), que se encuentra en cantidades abundantes en el cultivo de brécol (Traka y Mithen, 2009). No obstante, el efecto beneficioso de estos compuestos no queda limitado al SFN ya que otros ITCs derivados de otros GLS han mostrado tener también un efecto protector contra diferentes tipos de cáncer como es el caso del alil isotiocianato (AITC) (Xiao et al., 2003; Zhang et al., 2003; Hwang y Lee, 2006; Hwang y Kim, 2009), del fenetil isotiocianato (PEITC) (Adam Rodwell et al., 1993; Kenney et al., 2000; Conaway et al., 2005; Rose et al., 2005; Basu y Haldar, 2008), del indol-3-carbinol (I3C) (Higdon et al., 2007; Weng et al., 2008; Agerbirk et al., 2009) y del benzyl isotiocianato (BITC) (Zhang y Talalay, 1994; Fahey et al., 1997b; Kuang y Chen, 2004).

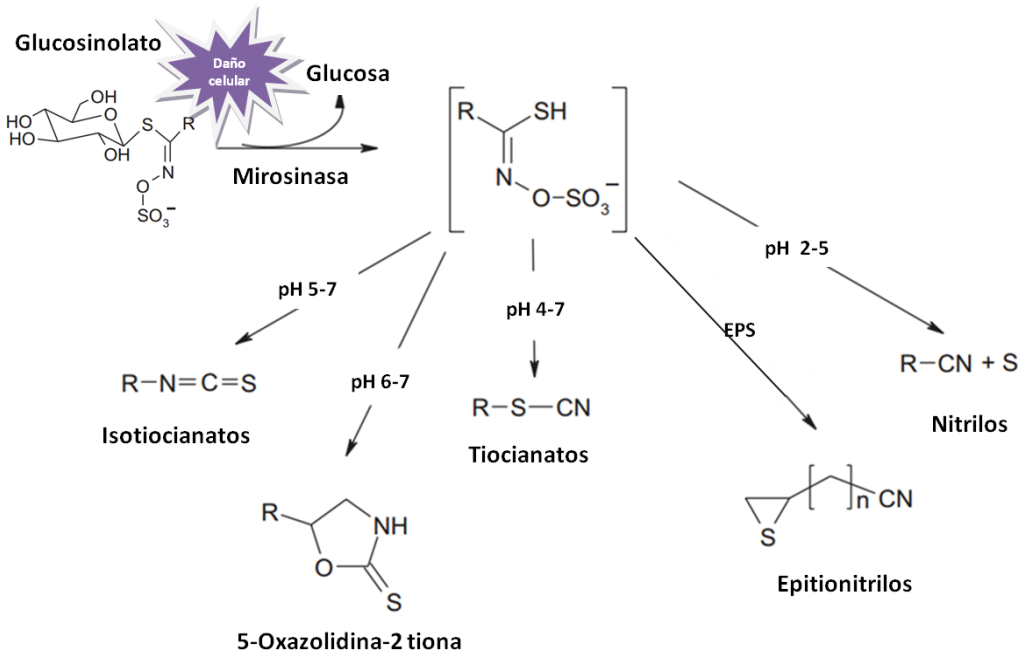


Figura 1.9: Hidrólisis de los glucosinolatos por las distintas isoformas de la enzima mirosinasa y los principales productos de hidrólisis. Extraído y modificado de Yan y Chen (2007) y Redovnikovic et al., (2008).

Debido a las propiedades biológicas de los GSLs, relacionadas con la defensa de la planta frente a estreses y con la salud humana, se están realizando importantes esfuerzos para localizar los principales genes responsables de su síntesis en distintas especies del género *Brassica* y así poder llegar a conocer de un modo más exhaustivo las vías de síntesis y acumulación de estos metabolitos, para poder manipular e incrementar el contenido de aquellos GSLs de interés y beneficiosos para la salud humana. Para poder identificar los genes implicados en la ruta de biosíntesis de GSLs, existen diferentes técnicas moleculares. Entre ellas se encuentra el análisis de QTLs, herramienta que nos permite detectar zonas genómicas que controlan la variación fenotípica de un carácter, en este caso el contenido en GSLs individuales. El principio del análisis de QTLs es simple y se basa en las diferencias únicas y precisas que revelan los marcadores moleculares, que se pueden genotipar y mapear fácilmente en poblaciones segregantes. Este análisis depende del desequilibrio de ligamiento entre un gen o genes que afectan a una variable

cuantitativa, de modo que las diferencias en los genotipos con los mismos marcadores están asociadas con diferentes fenotipos del carácter.

Un análisis de este tipo, aparte de aumentar el conocimiento sobre la herencia y la arquitectura genética de un carácter cuantitativo, facilita la identificación de marcadores que se pueden utilizar como herramientas indirectas de selección en un programa de mejora. En las dos últimas décadas, la capacidad de transferir regiones de interés utilizando marcadores moleculares ha dado lugar a multitud de estudios de mapeo de QTLs en la mayoría de cultivos de importancia económica, con miras a desarrollar marcadores para su empleo en selección asistida por marcadores o facilitar su clonación (Salvi y Tuberosa, 2005). A pesar de que *B. oleracea* es una de las especies más importantes desde el punto de vista económico del género *Brassica*, hasta la fecha no se ha publicado ningún estudio acerca del mapeo de QTLs para contenido en GSLs en esta especie, aunque sí se han realizado este tipo de estudios en otras especies de la familia *Brassicaceae* como *A. thaliana* (Kliebenstein et al., 2001b) o *B. napus* (Feng et al., 2012). La variación fenotípica para el contenido en GSLs en *B. napus* está controlada por diversos QTLs y también por interacciones epistáticas entre ellos, como demostró Feng et al. (2012). Por tanto, en esta tesis también sería interesante conocer si en *B. oleracea* existen este tipo de interacciones entre QTLs.

1.3.2.3. Modificación de la concentración de GSLs

En muchos cultivos hortícolas, los programas de mejora genética han atendido principalmente a aspectos como la productividad, resistencia a enfermedades, uniformidad del producto y la calidad externa, haciéndose poco énfasis en el contenido en compuestos beneficiosos para la salud. Sin embargo, en los últimos años, existe un interés creciente por parte de los consumidores en productos vegetales más sanos y que protejan frente a enfermedades. Es por ello que los programas de mejora genética van paulatinamente incorporando la mejora del contenido en compuestos beneficiosos para la salud humana y que prevengan enfermedades (calidad nutracéutica) entre sus objetivos. Algunos ejemplos de alimentos funcionales son los enriquecidos con determinadas vitaminas, minerales, fibra alimenticia o ácidos grasos y los alimentos a los que se les ha

añadido sustancias biológicamente activas, como los fitoquímicos y otros antioxidantes. Actualmente, en España, ya están disponibles en el mercado unos 200 productos de este tipo, sobre todo pan, cereales, lácteos, zumos y sal; gracias a ello, la industria alimentaria puede proveer alimentos con composición físico-química controlada o modificada, según el objetivo que se desea obtener con su ingesta. En este caso, sería de gran interés la obtención de nuevos cultivos con perfiles de GSLs modificados con el fin de incrementar aquellos beneficiosos sobre la salud humana (D'Mello et al., 1991; Fahey et al., 1997a; Rosa et al., 1997; Fahey y Stephenson, 1999; Fahey y Talalay, 1999; Cartea y Velasco, 2008; Forte et al., 2008; Van Horn et al., 2008; Virgili y Marino, 2008) y reducir los perjudiciales como el producto de degradación de la PRO, que puede causar bocio en animales (Liu et al., 2012). Por lo tanto, el incremento del contenido en GSLs con beneficios para la salud y la reducción en el contenido de los GSLs perjudiciales o tóxicos ha sido uno de los principales objetivos dentro de la mejora de los cultivos del género *Brassica*.

Los primeros intentos en la modificación de la composición de GSLs en cultivos de brásicas tuvieron lugar en los años 70. Por medio de introgresiones y mejora convencional, se obtuvieron las primeras variedades de colza 'doble cero' (cero contenido en ácido erúcico y cero contenido en GSLs) como la denominada 'Tower' en Canadá (Stefansson y Kondra, 1975) y una variedad con solo un 2% de dicho ácido como la llamada 'Erglu' en Alemania (Röbbelen y Thies, 1980). El aceite de colza o 'canola' extraído, así como las harinas obtenidas después de la extracción del aceite, pudieron ser usados de esta manera para la alimentación animal. Posteriormente, en los años 90 se inició un programa de mejora en el John Innes Centre (Reino Unido) con el fin de obtener variedades de brécol con alto contenido en 4-methylsulphinylbutyl (glucorrafanina, GRA). Se llevaron a cabo cruzamientos entre la especie silvestre *Brassica villosa*, (con un alto contenido en GRA) y variedades de brécol convencionales. Tras varios años de selección y cruzamientos mediante mejora tradicional se obtuvieron variedades de brécol con un alto contenido en GRA de 2 a 3 veces más alto comparado con las variedades de brócoli convencionales (Mithen et al., 2003; Sarikamis et al., 2006). Actualmente, la variedad de brócoli comercial denominada 'Beneforte super broccoli', enriquecida en GRA, y puesta en el mercado en 2011 se comercializa en toda Europa. Más recientemente, otros estudios, como el llevado a cabo mediante transgénesis por Li et al. (2003) en *A. thaliana*, consiguieron aumentar la concentración de algunos GSLs como

(GRA y glucoiberina, GIB) mediante el clonaje de un gen proveniente de otro cultivar. Recientemente, Liu et al. (2012) usando RNAi en la ruta de biosíntesis de GSLs obtuvieron semillas de *B. napus* enriquecidas en GRA silenciando el locus GSL-ALK. Estos trabajos, proponen la manipulación genética como una técnica con muchísimas aplicaciones en este campo, pero con una gran limitación ya que, actualmente en Europa todavía los cultivos y alimentos transgénicos no están aceptados socialmente.

Hasta ahora, la modificación en el contenido de GSLs se ha realizado mediante introgresiones o mediante transformación genética; sin embargo, no se ha intentado modificar el contenido en GSLs individuales aprovechando la variabilidad genética natural que existe dentro de los propios cultivos de brásicas mediante métodos de selección. El contenido en GSLs muestra una gran variabilidad entre diferentes variedades de una misma especie y cultivo, entre plantas de una misma variedad (Becker et al., 1999; Velasco et al., 2007) y entre órganos dentro de una misma planta. Así, la concentración de GSLs es mayor en los tejidos reproductivos (flores y semillas) que en los tejidos vegetativos (Bennet et al., 2004). Esta variabilidad genética permitirá modificar el perfil de GSLs, aumentando o reduciendo la concentración de un GSL en particular mediante la selección de aquellos genotipos con el fenotipo deseado.

Existen diferentes métodos de selección para modificar la composición del carácter deseado incluyendo la selección masal, la selección individual, la selección por familias de medios hermanos, la selección por familias de hermanos completos y la selección recurrente, entre otras. Teniendo en cuenta que *B. oleracea* es un cultivo alógamo y que los GSLs son caracteres que han mostrado una elevada heredabilidad (Van Doorn et al., 1998; Márquez-Lema et al., 2009; Madsen et al., 2014), esta tesis plantea la realización de una selección masal por el contenido en diferentes GSLs individuales como un método sencillo y eficaz para modificar la composición en estos compuestos. Este procedimiento es el más sencillo y consiste en elegir los mejores individuos (en base al carácter por el que se está seleccionando) que se recombinarán entre sí, y cuyas semillas, mezcladas, constituirán la generación siguiente. El proceso se repite un número determinado de ciclos de selección y recombinación hasta que se fija el carácter deseado repitiendo el proceso durante varias generaciones (Figura 1.10).

Este método ha sido practicado por los agricultores a lo largo de los siglos para mejorar características deseables en diferentes cultivos y aún se utiliza hoy en día en los

programas de mejora genética cuando es esencial la rapidez del proceso y cuando un carácter tiene una alta heredabilidad. En el caso de los cultivos de B. oleracea, la selección masal sería además el método más sencillo de aplicar dado el carácter alógamo de la especie y la dificultad de realizar cruzamientos manuales. Para determinar si este método es válido, tanto para incrementar como para disminuir el contenido en un GSL en concreto, se propone realizar una selección masal divergente. Este tipo de selección permitirá evaluar en una población el efecto de la selección en sentidos opuestos, cuando existe suficiente varianza genética, obteniéndose al final dos poblaciones idénticas pero con diferentes frecuencias de aquellos genes determinantes de la característica de interés.

Existe un trabajo previo, en el cual se utilizó un programa de selección divergente para modificar el contenido total de GSLs en hojas de una variedad de ciclo rápido de *B. rapa* (Stowe y Marquis, 2011). Tras tres ciclos de selección, se encontraron diferencias significativas entre los genotipos altos y los bajos con respecto al genotipo control. En cambio, este estudio pretende llevar a cabo la modificación de tres GSLs mayoritarios presentes en berzas (*Brassica oleracea* var. *acephala*), dos de ellos alifáticos, sinigrina (2-propenil, SIN) y glucoiberina (3-methilsulfinilpropil, GIB) y uno indólico (3-indolilmetil, GBS) con rutas de síntesis diferentes para lo cual se desarrollaron tres programas de selección diferentes e individuales.

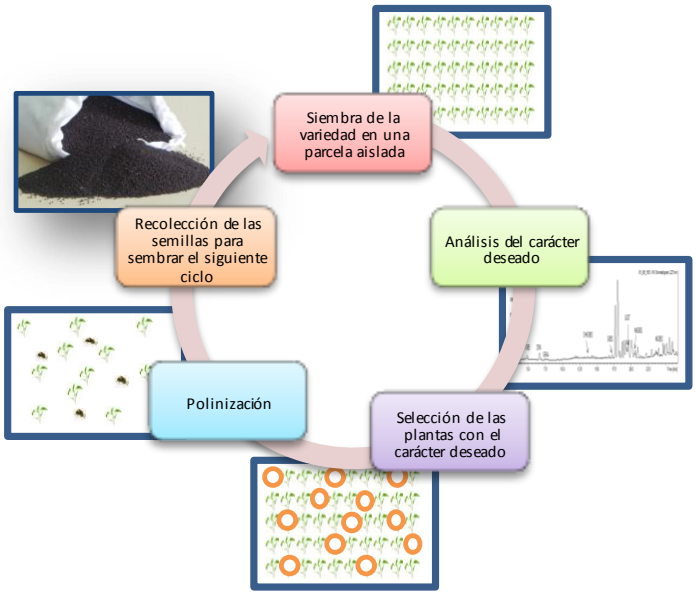


Figura 1.10: Ejemplo de selección masal en alógamas.

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CAPÍTULO II

JUSTIFICACIÓN DEL TRABAJO Y OBJETIVOS

2. JUSTIFICACIÓN DEL TRABAJO Y OBJETIVOS

2.1. JUSTIFICACIÓN DEL TRABAJO

Un gran número de estudios epidemiológicos han demostrado que un consumo regular de hortalizas de la familia de las brásicas ayuda a la disminución del riesgo de padecer diferentes tipos de cáncer y otras enfermedades crónicas incidiendo favorablemente en la mejora de calidad de vida de las personas. Este efecto beneficioso ha sido atribuido en su mayoría a la presencia de metabolitos secundarios con diferentes propiedades biológicas entre los que destacan antioxidantes naturales tales como la vitamina C, compuestos fenólicos y los GSLs, cuya presencia en la naturaleza se encuentra limitada, prácticamente a los vegetales de la familia *Brassicaceae*.

La obtención de cultivos de brásicas enriquecidos en este tipo de compuestos bioactivos exige en primer lugar el conocimiento de su control genético. Hasta la fecha, existen numerosos estudios acerca de la actividad antioxidante de los diferentes cultivos de brásicas, pero en cambio se desconoce la base genética de estos compuestos. Los GSLs también han sido objeto de múltiples estudios acerca de cómo las variables ambientales, bióticas y el procesado afectan a su contenido final en la planta. Aunque ya se ha avanzado en las investigaciones para llegar a conocer las bases genéticas de su síntesis y se han identificado algunos de los genes principales, todavía se desconocen muchos de los genes implicados en la síntesis de estos compuestos así como la regulación de esta ruta.

En los últimos años ha habido un interés creciente por parte de los consumidores en alimentos más sanos y que protejan frente a distintas enfermedades; es por esto, que los programas de mejora han ido incorporando ente sus objeticos la mejora del contenido de estos compuestos bioactivos. Además, la obtención de un material que solo difiera en el contenido en un GSL en particular permitiría llevar a cabo diferentes estudios acerca de sus efectos biológicos.

2.2. OBJETIVOS

El principal objetivo de la presente Tesis Doctoral es ampliar el conocimiento sobre los mecanismos o los genes implicados en la síntesis y acumulación de compuestos beneficiosos para la salud humana como son los compuestos GSLs y los compuestos con actividad antioxidante presentes en los cultivos de *Brassica oleracea*.

Para ello se plantean los siguientes objetivos parciales:

- Estudio y búsqueda de QTLs relacionados con la actividad antioxidante en *B. oleracea* como un paso previo a identificar posibles genes candidatos relacionados con esta. Paralelamente, se estudiará la relación entre los diferentes métodos para medir la actividad antioxidante así como la relación entre estos métodos y el contenido en compuestos fenólicos, carotenoides y antocianinas.

- Estudio y búsqueda de QTLs relacionados con la acumulación y composición de glucosinolatos en diferentes órganos de *B. oleracea.* A partir de ellos se buscarán genes candidatos y se determinarán las relaciones epistáticas entre los diferentes QTLs.

- Evaluación de la eficacia de la selección divergente para el contenido de tres glucosinolatos mayoritarios en hojas en una población de *B. oleracea*. Paralelamente, se observará el efecto colateral en la modificación de glucosinolatos en otros órganos de la planta y se determinará si el contenido en otros glucosinolatos puede verse alterado por la selección.

CAPÍTULO III

IDENTIFICATION OF ANTIOXIDANT CAPACITY-RELATED QTLs IN *BRASSICA OLERACEA*

3. IDENTIFICATION OF ANTIOXIDANT CAPACITY-RELATED QTLS IN *BRASSICA OLERACEA*

Tamara Sotelo, María Elena Cartea, Pablo Velasco, Pilar Soengas

Group of Genetics, Breeding and Biochemistry of Brassicas, Department of Plant Genetics, Misión Biológica de Galicia, Spanish Council for Scientific Research (MBG-CSIC), Pontevedra, Spain.

3.1. ABSTRACT

Brassica vegetables possess high levels of antioxidant metabolites associated with beneficial health effects including vitamins, carotenoids, anthocyanins, soluble sugars and phenolics. Until now, no reports have been documented on the genetic basis of the antioxidant activity (AA) in Brassicas and the content of metabolites with AA like phenolics, anthocyanins and carotenoids. For this reason, this study aimed to: (1) study the relationship among different electron transfer (ET) methods for measuring AA, (2) study the relationship between these methods and phenolic, carotenoid and anthocyanin content, and (3) find QTLs of AA measured with ET assays and for phenolic, carotenoid and anthocyanin contents in leaves and flower buds in a DH population of *B. oleracea* as an early step in order to identify genes related to these traits.

Low correlation coefficients among different methods for measuring AA suggest that it is necessary to employ more than one method at the same time. A total of 19 QTLs were detected for all traits. For AA methods, seven QTLs were found in leaves and six QTLs were found in flower buds. Meanwhile, for the content of metabolites with AA, two QTLs were found in leaves and four QTLs were found in flower buds. AA of the mapping population is related to phenolic compounds but also to carotenoid content. Three genomic regions determined variation for more than one ET method measuring AA. After the syntenic analysis with *A. thaliana*, several candidate genes related to phenylpropanoid biosynthesis are proposed for the QTLs found.

Keywords: anthocyanin content, ABTS, Folin, FRAP, DPPH, carotenoid content, phenolic compounds, antioxidant activity.

3.2. INTRODUCTION

Brassicaceae plants represent one of the major vegetable crops grown worldwide, with *Brassica oleracea* L. (2n = 18) as the main *Brassica* species consumed in Europe and the USA. Cruciferous vegetables, in particular those included in the *Brassica* genus, are an important part of the diet as they provide a multitude of nutrients and bioactive compounds (Liu, 2004). A high consumption of Brassica vegetables reduces the risk of age-related chronic illnesses, degenerative diseases (Kris Etherton, 2002) and several types of cancer (Wang et al., 2004). Human health benefits associated to *Brassica* consumption could be attributed, in part, to the large amount of constituents having strong antioxidant activity (AA). In fact, AA of Brassica vegetable extracts is higher compared to that of other vegetable crops like green pepper, carrot, potato or green bean (Cao et al., 1996). Antioxidants have long been recognized to have protective functions against oxidative damage and are associated with a reduced risk of chronic diseases (Okarter and Liu, 2010). *Brassica* vegetables possess high levels of antioxidant metabolites associated with beneficial health effects, including vitamins (especially vitamin A, C, E, K and B-6), carotenoids (such as γ- and β-carotene and zeaxanthin), anthocyanins, folate, soluble sugars and phenolic compounds which are known to be the major antioxidants of Brassica crops (Dekker et al., 2000; Ou et al., 2002; Vallejo et al., 2002; Powers et al., 2004; Vallejo et al., 2004a; Heimler et al., 2006; Zhou and Yu, 2006; Podsedek, 2007; Okmen et al., 2011).

Due to the complexity of food composition, separating each antioxidant compound and studying it individually is costly and inefficient. In addition, there might be synergistic interactions among the antioxidant compounds (Huang et al., 2005). There are numerous methods for measuring the total AA of a plant extract *in vitro*. The 2- single electron transfer reaction based assays (ET) measure the reducing capacity of the samples. The ET group includes different methods like the ferric ion reducing antioxidant power assay (FRAP), and the AA measured with the reagents ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and DPPH (2, 2-diphenyl-1-picrylhydrazyl), among others (Huang et al., 2005). Generally speaking, correlations found among these three methods are high in brassica extracts. Soengas et al. (2012) found that the correlation between DPPH and FRAP was 0.8 when analyzing several *B. oleracea* crops. Kusznierewicz et al. (2008) found a correlation of 0.96 between ABTS and DPPH in

white cabbage. Zhi-Xiang et al. (2011) found correlations ranging from 0.76 to 0.82 among the three cited methods when analyzing different vegetables, including broccoli. In most studies, several ET methods are often used in order to measure the AA of a sample, but theoretically it could be possible to choose only one because of the high correlations among assays.

Phenolic compounds are known to be the major group with antioxidant capacity in brassica crops (Podsedek, 2007). These compounds are able to scavenge reactive oxygen species due to their electron donating properties. The most widespread and diverse group of polyphenols in *Brassica* species are flavonoids and hydroxycinnamic acids. In many *in vitro* studies, phenolic compounds demonstrated higher AA than other antioxidants, such as vitamins and carotenoids (Vinson et al., 1995).

Several studies have demonstrated that highly pigmented cultivars of some vegetables (i.e. cabbage, cauliflower) possess stronger AA than their respective light-colored cultivars (Gajewski et al., 2007; Li et al., 2012; Pace et al., 2013). This could indicate that pigments '*per se'* have AA. Carotenoids are a diverse group of more than 600 natural pigments that accumulate in the plastids of some vegetables leaves, flowers and fruits (Paiva and Russell, 1999). Some carotenoids are essential nutrients for humans, while others have protective effects against several diseases. Anthocyanins are natural pigments responsible for the blue, purple, red and orange colors in the major parts of all higher plants and have attracted much interest due to their impact on the sensorial characteristics of food products, as well as their health-related properties through various biological activities (He et al., 2010; Yang et al., 2011). The AA of brassica crops has been mainly related to phenolic compounds and vitamin C. However, carotenoids and anthocyanins could also play an important role.

Comparisons of *in vitro* AA of the main *B. oleracea* crops demonstrated that broccoli, kale and red cabbage show high AA (Podsedek et al., 2006; Kusznierewicz et al., 2008). Soengas et al. (2012) compared the AA of six brassica crops, including broccoli, cabbage, cauliflower, kale, nabicol and tronchuda cabbage, at four different plant stages with DPPH and FRAP assays. They found that kale and broccoli had the highest AA. Nilson et al. (2006) found that AA of curly kale was at least 10-fold higher than that of cauliflower and white cabbage. At present, there are many studies about AA of brassica crops because of the health related properties of antioxidants. However, as far

as we know, there are no reports about genetics and heredity associated with AA in the *Brassica* genus.

QTL analysis is a very important tool in order to study the genetic base of AA. For the last decades, quantitative trait mapping has been the most common approach in order to analyze complex traits and measure the association of genetic markers with phenotypic variation. Identification of QTLs is essential for the understanding of the quantitative genetic control of AA and it is an early step in order to identify and estimate the gene number controlling each trait variation. The high co-linearity between *A. thaliana* and *Brassica* species can be used for identifying candidate genes underlying QTLs that affect AA. To our knowledge, this is the first report on the genetic basis of AA in brassica crops. In other crops, only Jin et al. (2009) in rice, Dobson et al. (2012) in raspberry and Hayashi et al. (2012) in lettuce studied QTLs for total water soluble AA and total phenolic, anthocyanin and carotenoid contents.

For this reason, the aims of our research were 1) to study the relationship among different ET methods for measuring AA, 2) to study the relationship between these methods and phenolic, carotenoid and anthocyanin contents and 3) to find QTLs of AA measured with ET assays and for phenolic, carotenoid and anthocyanin contents in two organs of a DH population of *B. oleracea* as an early step in order to identify genes related to these traits.

3.3. MATERIAL AND METHODS

3.3.1. Chemicals

DPPH (2,20-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-striazine), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), hydrochloric acid, phenolics reagent, ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)), potassium persulphate and gallic acid were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); ferric chloride and methanol were obtained from Panreacquimica S.A. (Castellar del Vallés, Spain).

3.3.2. Plant material and growing environments

The double haploid (DH) mapping population employed in this study (BolTBDH) was created from an F_1 individual, derived by crossing a DH broccoli line 'Early Big'(P_2) and a DH rapid cycling of Chinese kale line (TO1000DH3,P_1) (Iñiguez-Luy et al., 2009). Parents and 155 DH lines were grown in autumn 2011 (from September to November) and stored in the greenhouse under controlled conditions: 16 h of daylight and a temperature of 24 ± 2 °C; 8 h of darkness having 18 ± 2ºC at night; and a relative humidity of 55% in order to obtain enough seed in the same environmental conditions (Figure 3.1). Plants were sown in a completely randomized experiment with two replications and four plants per replication. Two sample types were collected and analysed: leaves (one month after sowing) and flower buds (taken sequentially depending on the maturity of each line). Bulks of individual samples were taken from each replication. Samples were frozen *in situ* in liquid N_2, immediately transferred to the laboratory and frozen at -80 °C. All samples were freeze-dried (BETA 2-8 LD plus, Christ) for 72 h. The dried material was powdered by using an IKA-A10 (IKA-Werke GmbH & Co.KG) mill, and the fine powder was used for methanolic extractions.

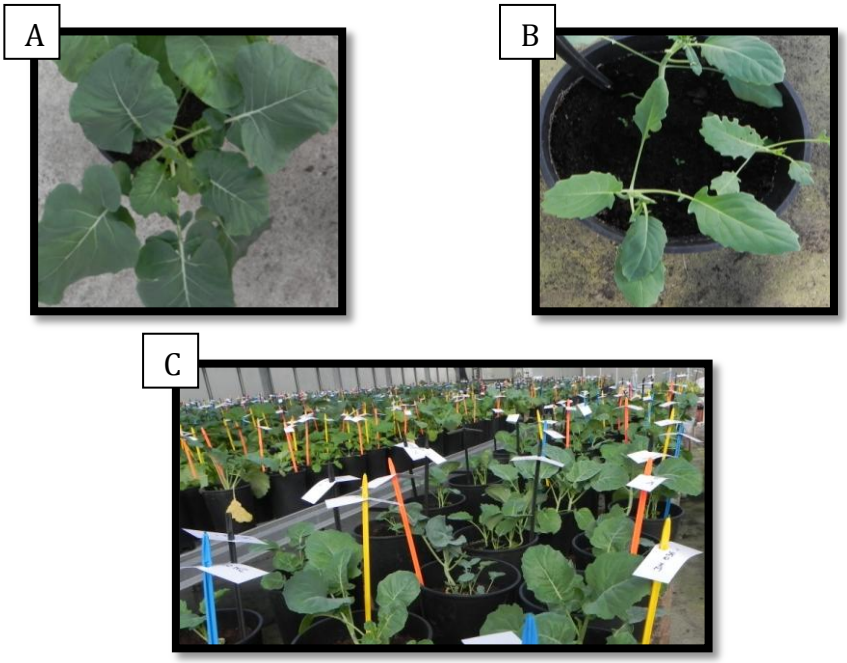


Figura 3.1: Parental Early Big (A), parental rapid cycling TO1000DH3 (B) and lines of DH population.

3.3.3. Evaluation of AA

Freeze-dried and ground samples (10 mg) were extracted with 1 ml of 80% aqueous methanol in dark maceration for 24h. After centrifugation (3700 rpm, 5 min), methanolic extracts were employed in order to determine AA (FRAP, DPPH and ABTS) of the mapping population. All AA assays and the content of metabolites with AA were carried out spectrophotometrically by using a microplate spectrophotometer (Spectra MR; Dynex Technologies, Chantilly, VA). Two repetitions were made for each sample and analysis. Standards prepared with different concentrations of Trolox® (0, 0.008, 0.016, 0.024, 0.032, 0.04 mM) were measured for FRAP, DPPH and ABTS analyses and AA values were normalized to Trolox® equivalents per gram of dry weight (Figure 3.2).

3.3.3.1. FRAP assay

The ferric reducing antioxidant activity (FRAP) assay of Benzie and Strain (1996) was measured in all samples. Fresh FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6), one volume of 10 mM TPTZ in 40 mM hydrochloric acid and one volume of 20 mM ferric chloride, and then incubating at 37 ºC for 5 minutes. For each analysis, 30 µl of methanolic solution of the two organs (leaves and flower buds) were added to 20 µl of distilled water and 250 µl of fresh FRAP solution and mixed thoroughly. The increase in absorbance was recorded at 593 nm after 20 min.

3.3.3.2. DPPH radical scavenging activity

The antioxidant activity by the DPPH method was determined by monitoring the disappearance of the radical DPPH spectrophotometrically, according to Brand-Williams et al. (1995). The working DPPH reagent was prepared by dissolving DPPH in methanol to a final concentration of 75 µM. Fifty microliters of extract for leaves and 35µl for flower buds were added to 250 µl of freshly prepared DPPH reagent and mixed thoroughly. Readings were taken at 517 nm after 30 min of incubation in the dark at room temperature.

3.3.3.3. ABTS+ radical scavenging activity

The method of decolorization of free radical ABTS+ employed was a modified version of that used by Samarth et al. (2008) and initially reported by Re et al. (1999). ABTS+ was generated by oxidation of ABTS 7 mM with potassium persulphate 2.45 mM in water, at room temperature for 16 h. For each analysis, the ABTS+ solution was freshly diluted with water in order to obtain an initial absorbance around 0.8 at 734 nm. An aliquot of 20 µl methanolic extract for leaves and 30µl for flower buds were added to 250 µl of ABTS+ solution. Absorbances were measured at 734 nm after 30 min of incubation in the dark at room temperature.

3.3.3.4. Quantification of phenolic content

The total phenolic content of the extracts was determined according to the phenolic colorimetric method described by Dewanto et al. (2002). The same methanolic extracts employed for AA assays were employed in order to determine phenolic content. Extracts were oxidized with 50 µl of 0.5 M Folin reagent. After 5 min, 200 µl of a 20% Na_2CO_3 solution were added in order to neutralize the reaction. Absorbances were measured at 760 nm after 2h of incubation in the dark at room temperature. Standards prepared with different concentrations of gallic acid (0, 0.008, 0.016, 0.024, 0.032 and 0.04 mM) were also measured. Results were expressed in terms of micromoles of gallic acid equivalents per gram of dry weight.

3.3.3.5. Quantification of carotenoid content

Carotenoid content was determined according to Sims & Gamon (2002) with minor modifications. Lyophilized samples (10 mg) were ground in 1 ml cold acetone / Tris buffer solution (80:20 vol:vol, pH = 7.8). Samples were mixed overnight in the dark at room temperature; afterwards, the absorbance of samples was measured at 537, 647 and 663 nm. Carotenoid content was computed by following the equations of Sims & Gamon (2002) and results were expressed in micromoles per gram of dried weight.

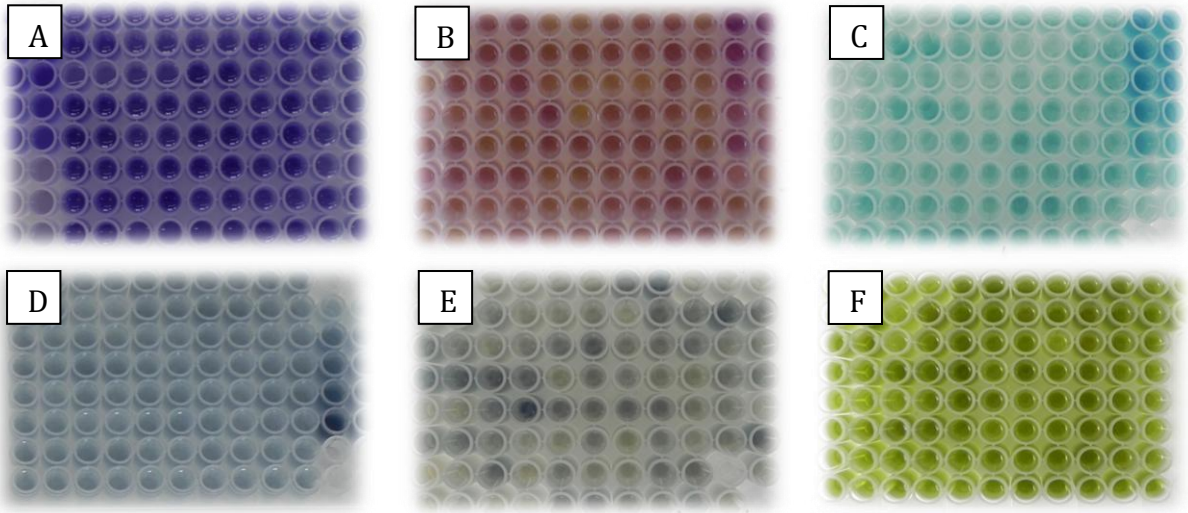


Figure 3.2: Colored reaction of the different methods employed to evaluated the AA, FRAP (A), DPPH (B) and ABTS (C), and the content of total phenolics (D), anthocyanins (E) and carotenoids (F).

3.3.3.6. Quantification of anthocyanin content

Anthocyanin content was determined according to Murray et al. (1991) with minor modifications. Lyophilized samples (10 mg) were ground in 1 ml of cold methanol / HCL / water (90:1:1, vol:vol:vol). Samples were mixed overnight in the dark at room temperature. The absorbance of samples was measured at 529 and 650 nm and anthocyanin content was determined by using the equation described in Sims & Gamon (2002). Results were expressed in micromoles per gram of dried weight.

3.3.4. Statistical and QTL analysis

A combined analysis of variance across organs and individual analyses of variance for each organ were made for the AA content measured ABTS, DPPH, FRAP assays and for phenolic, carotenoid and anthocyanin contents by using the procedure ANOVA of SAS v

9.2 (SAS, 2008). Parental differences were analyzed one-tail "t" test by using PROC TTEST of SAS v 9.2 (SAS, 2008). Simple correlation coefficients were computed with PROC CORR of SAS v 9.2 (SAS, 2008) for each trait.

The genetic map created by Iñiguez-Luy et al. (2009) has 279 markers (SSRs and RFLPs) distributed along nine linkage groups (C1-C9) with a total distance of 891.4 cM and a marker density of 3.2 cM / marker. Quantitative trait locus mapping was carried out through a composite interval mapping method (Zeng, 1994) by using PLABQTL (Utz and Melchinger, 2003). Individual analyses were carried out for each trait and organ (leaves and flower buds). A likelihood odds (LOD) threshold was chosen for each trait in order to declare the putative QTL significant by following a permutation test, with N=1000, and a critical alpha value of 25%. The confidence intervals were set to 95%. The analysis and cofactor election were carried out by following PLABQTL's recommendations, using an 'F-to-enter' and an 'F-to-delete' value of 7.

The proportion of phenotypic variance explained for a specific trait was determined by the adjusted coefficient of determination of regression (R^2) fitting a model which includes all detected QTLs (Papst et al., 2004). Fivefold cross-validation of QTLs was performed by following the procedures described by Utz et al. (2000). The whole data set was randomly split into k = 5 data subsets. Four of these subsets were combined to form the estimation set (ES). The remaining subset formed the test set (TS), in which predictions derived from ES were tested for their validity by correlating predicted and observed data. We used 1,000 replicate CV/G runs. Estimates of medians and percentiles and the frequency of QTL detection in ES and TS were calculated over all replicated CV/G runs. The frequency of QTL detection gives us an estimation of the precision of QTL localization. The PLABQTL (Utz and Melchinger, 2003) software package was used for all calculations.

Iñiguez-Luy et al. (2009) identified collinear genomic blocks between the BolTBDH mapping population and *A. thaliana* by using a synteny analysis. This information was employed in order to locate candidate genes which may directly account for QTLs in *B. oleracea.* By following this approach, we searched in the database TAIR (the *Arabidopsis* information resource http://www.arabidopsis.org) genes related to phenylpropanoid biosynthetic process metabolism (phenolic compounds and anthocyanins are synthetized following this pathway) and genes involved in the

carotenoid biosynthetic process by including the words 'phenylpropanoid' and 'carotenoid' into the field 'description of the gene in TAIR. Twenty one genes related to phenylpropanoids and 24 genes related to carotenoids were found. We tried to locate these genes on the BolTBDH map by means of *in silico* mapping.

3.4. RESULTS

3.4.1. Quantitative variation for methods measuring AA and the content of metabolites with AA

In this study AA in leaves and flower buds was determined by three ET methods: FRAP, DPPH and ABTS. The content of metabolites with AA (phenolics, anthocyanins and carotenoids) was also determined. We used two ET methods (DPPH and ABTS) where the scavenging was followed by monitoring the decrease in absorbance over time, which occurred due to the AA of the sample (Fukumoto and Mazza, 2000). For the FRAP assay, the extract shows an increase of absorbance over time dependent on their AA (Nilsson et al., 2005). A transgressive distribution was found for all traits in both organs (Figure 3.3). Results obtained from each analysis are considered below.

3.4.1.1. FRAP, DPPH and ABTS assays

Mean values for the FRAP and DPPH methods in the population were lower than the corresponding values of ABTS assay in both organs (leaves and flower buds). In leaves, we found mean values of 18.36, 14.04 and 24.78 µmol Trolox g-1 DW in FRAP, DPPH and ABTS assays, respectively. In flower buds, we found values of 15.37, 12.51 and 25.16 µmol Trolox g-1 DW in FRAP, DPPH and ABTS assays, respectively (Table 3.1). Population mean values between the two organs present highly significant differences for FRAP ($F= 75.95$, $P =0.0129$) and DPPH ($F= 65.09$, $P =0.0150$) methods.

Table 3.1: Antioxidant activity of parents and population measured in leaves and flower buds with three different antioxidant assay systems and the content of three metabolites with antioxidant activity.

	Leaves			Flower buds		
Traits	**P1**	**P2**	**Population mean**	**P1**	**P2**	**Population mean**
ABTS (µmol Trolox g^{-1} DW)	42.06	44.89	24.78	21.13	30.94	25.16
DPPH (µmol Trolox g^{-1} DW)	20.20	34.18	14.04	50.65	47.84	12.51
FRAP (µmol Trolox g^{-1} DW)	48.17	56.27	18.36	59.40	28.71	15.37
PHENOLICS (µmol Gallic Acid g^{-1} DW)	8.02	8.91	3.64	5.55	5.54	4.14
ANTHOCYANINS (µmol g^{-1} DW)	0.03	0.67	58.53	0.04	0.13	13.31
CAROTENOIDS (µmol g^{-1} DW)	1.48	2.17	1.98	0.84	0.17	0.28

3.4.1.2. Metabolites with AA: phenolic, anthocyanin and carotenoid content

Concerning the content of metabolites with AA, we found two different profiles. For the phenolics assay, population showed higher mean values in flower buds than in leaves (4.14 and 3.64 µmol gallic acid g-1 DW, respectively), although differences were not significant. However, both parental lines had higher phenolic content in leaves than in flower buds (Figure 3.3).

Leaves of the mapping population had higher anthocyanin and carotenoid content (58.53 µmol g-1 DW and 1.98µmol g-1 DW, respectively) compared to flower buds (13.2131µmol g-1 DW and 0.28µmol g-1 DW, respectively). Mean anthocyanin content of the population represents a strong increase compared to the values found in both parents. As other assays previously described, anthocyanins presented transgressive distributions for both organs (Figure 3.3).

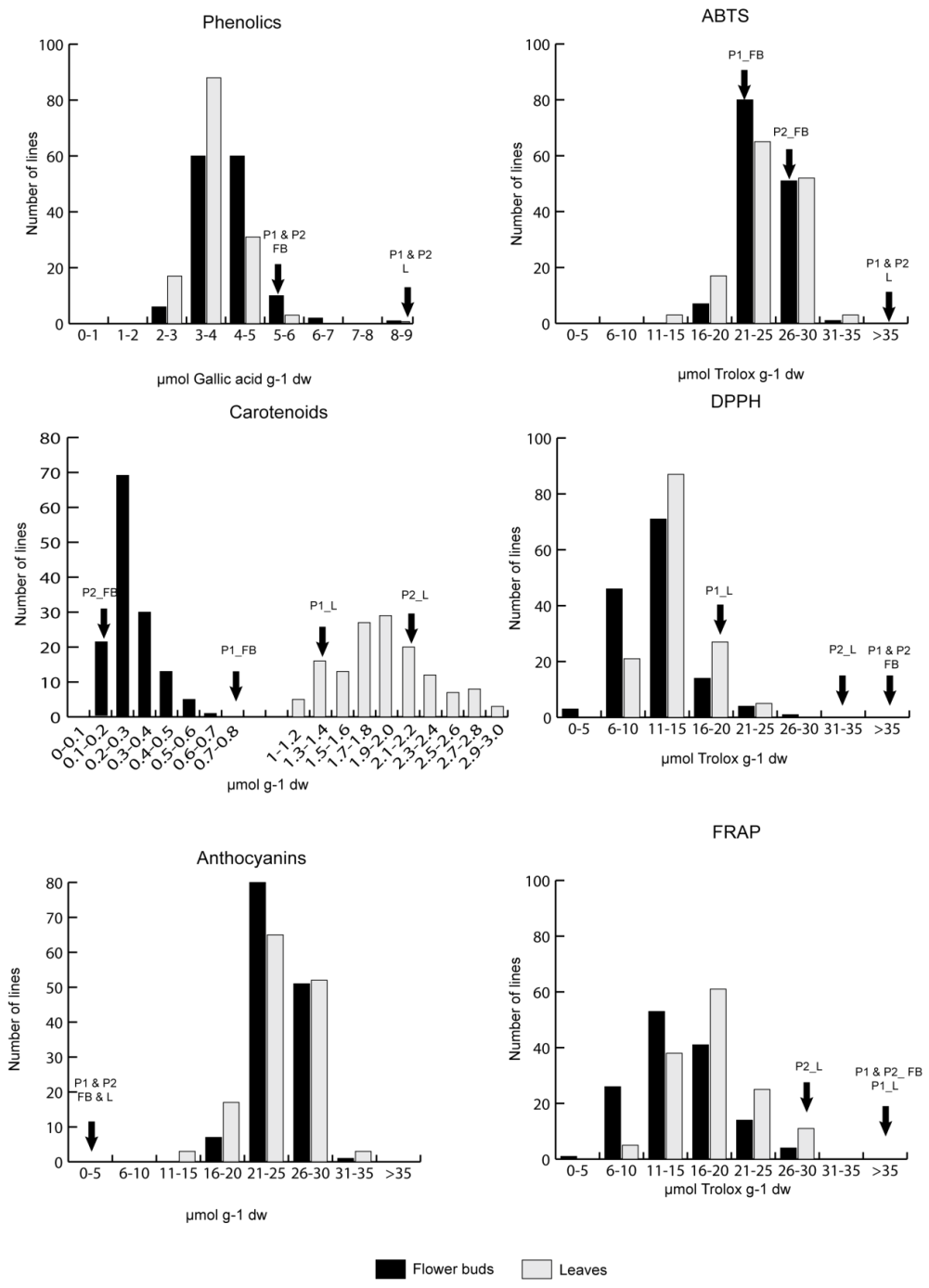


Figure 3.3: Distribution of the three metabolites with antioxidant activity, carotenoids, anthocyanins and phenolics and the three antioxidant assay methods, ABTS, DPPH and FRAP in the BolTBDH population. Arrows indicate values for the P1 (DH rapid cycling of Chinese kale TO1000DH3) and P2 (DH broccoli line 'Early Big') in the two organs under study, leaves (L) and flower buds (FB).

In the case of carotenoid content, differences between both organs were highly significant (F=80.44, P=0.012). Correlation coefficients among methods measuring AA, phenolic and pigment contents in the BolTBDH population were made. Pairwise correlations between AA measured with three ET assays (FRAP, DPPH and ABTS) were positive and highly significant (P≤0.01) for both leaves and flower buds in the correlation analysis carried out with all lines of the mapping population. However, correlation coefficients were moderately low (Table 3.2). The highest correlations occurred between DPPH and FRAP assays for both organs. The correlation values were 0.486 in flower buds and 0.526 in leaves.

Table 3.2: Correlation coefficients for leaves (above the diagonal) and flower buds (below the diagonal) between the three antioxidant assay methods and the content of three metabolites with antioxidant activity (n=280).

Leaves / Flower buds	**ABTS**	**FRAP**	**DPPH**	**Phenolics**	**Anthocyanins**	**Carotenoids**
ABTS	----	0.197**	0.267**	0.434**	-0.339**	0.140*
FRAP	0.189**	----	0.526**	0.151*	0.103	0.100
DPPH	0.389**	0.486**	----	0.250**	0.164**	0.051
Phenolics	0.633**	0.221**	0.227**	----	-0.110	0.086
Anthocyanins	-0.130*	-0.027	-0.076	-0.100	----	-0.081
Carotenoids	-0.165**	0.305**	0.005	-0.013	0.176**	----

* Significant at P≤0.05, and ** significant at P≤0.01. ABTS: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); FRAP: ferric ion reducing antioxidant power assay; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

On the other hand, correlation coefficients between the content of phenolic compounds and the three AA methods were positive and significant for both organs (P≤0.01). Significant correlations between the anthocyanin content with DPPH and ABTS

were found in leaves. Correlation with DPPH was positive; however, correlation with ABTS was negative (R=-0.339, P≤0.01) (Table 3.2). Anthocyanin content was significantly and negatively correlated to ABTS assay (Table 3.2). Carotenoid content showed significant correlation coefficients with the AA measured with ABTS assay (R=0.140, P≤0.05) in leaves, and significant and positive correlation coefficients with FRAP assay in flower buds (R=0.305, P≤0.01). Furthermore, correlation between carotenoids and ABTS assay was negative and highly significant in flower buds (R=-0.165, P≤0.01) (Table 3.2).

3.4.2. QTL mapping for methods measuring AA, phenolic and pigment contents in the BolTBDH population

A total of 19 QTLs were detected for all traits. The number of QTLs by linkage group ranged between one in C9 and five in C3 (Fig. 2). For methods measuring AA, seven significant QTLs were found in leaves. The value of R^2 ranged between 9.8% for FRAP in C3 and 17.4% for DPPH in C4, respectively (Table 3.3). Three of these QTLs had a frequency of cross-validation higher than 50%. In flower buds, six significant QTLs were found. R^2 value varied between 9.8% for ABTS in C6 and 12.1% for FRAP content in C3, but only two of the QTLs had a frequency of cross-validation higher than 50%.

For the content of metabolites with AA, two significant QTLs for phenolic content were found in leaves. The value of R^2 ranged between 10.3 and10.4% in C7 and all of them had a frequency of cross-validation higher than 50%. Meanwhile, four significant QTLs were found in flower buds. The value of R^2 ranged between 9.9 and 12.6% for carotenoids in C5 and C9, respectively. Only one of these QTLs presents a frequency of cross-validation higher than 50%. One QTL for anthocyanin content was found on C3 in flower buds, from which a R^2 value of 10.9% and three QTLs for carotenoid content were found on C5, C8 and on C9. R^2 values varied between 9.9 and 12.6% (Table 3.3).

Based on the position of QTLs and taking into account their confidence interval, three genomic regions determined variability for different traits. The genomic region located on C3, in the interval from marker pW125dE to fito156c & pW133cH (AA-C3),

determined variation for the three different methods measuring AA: FRAP in leaves and ABTS and DPPH in flower buds. A second genomic region on C7 from pW225aD to pW104aE (AA-C7) determined variation for the methods measuring AA (ABTS in leaves and FRAP in flower buds) and phenolic content in leaves. Alleles for increasing AA or phenolic content are given by P2 in both genomic regions on C3 and C7.

A third genomic region on C5 (AA-C5), from pW209aH to Na10-F06b & fito132a, also determined variation for the methods measuring AA (DPPH in leaves and ABTS in flower buds) and carotenoid content in flower buds. In this case, alleles for increasing AA and carotenoid content are given by P1.

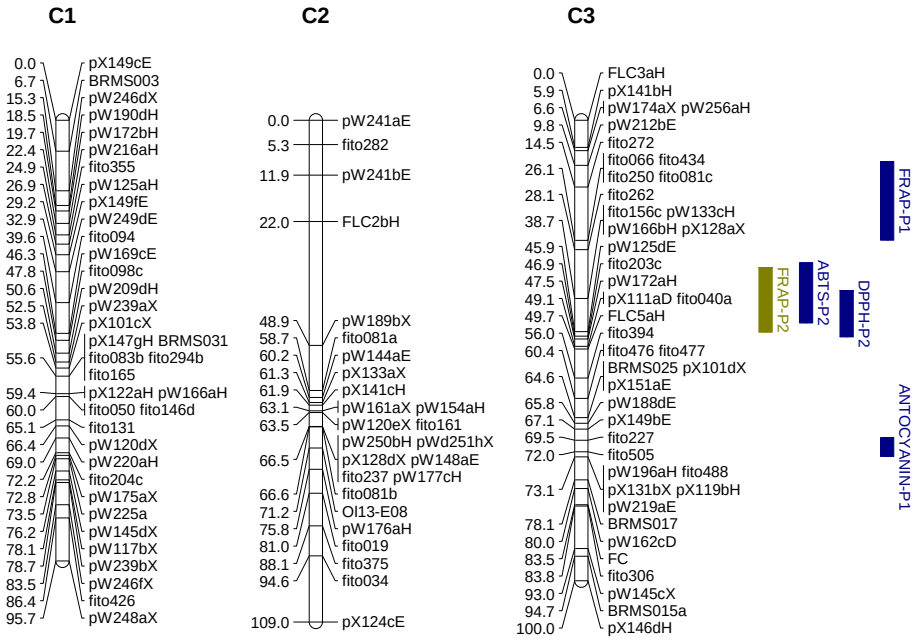
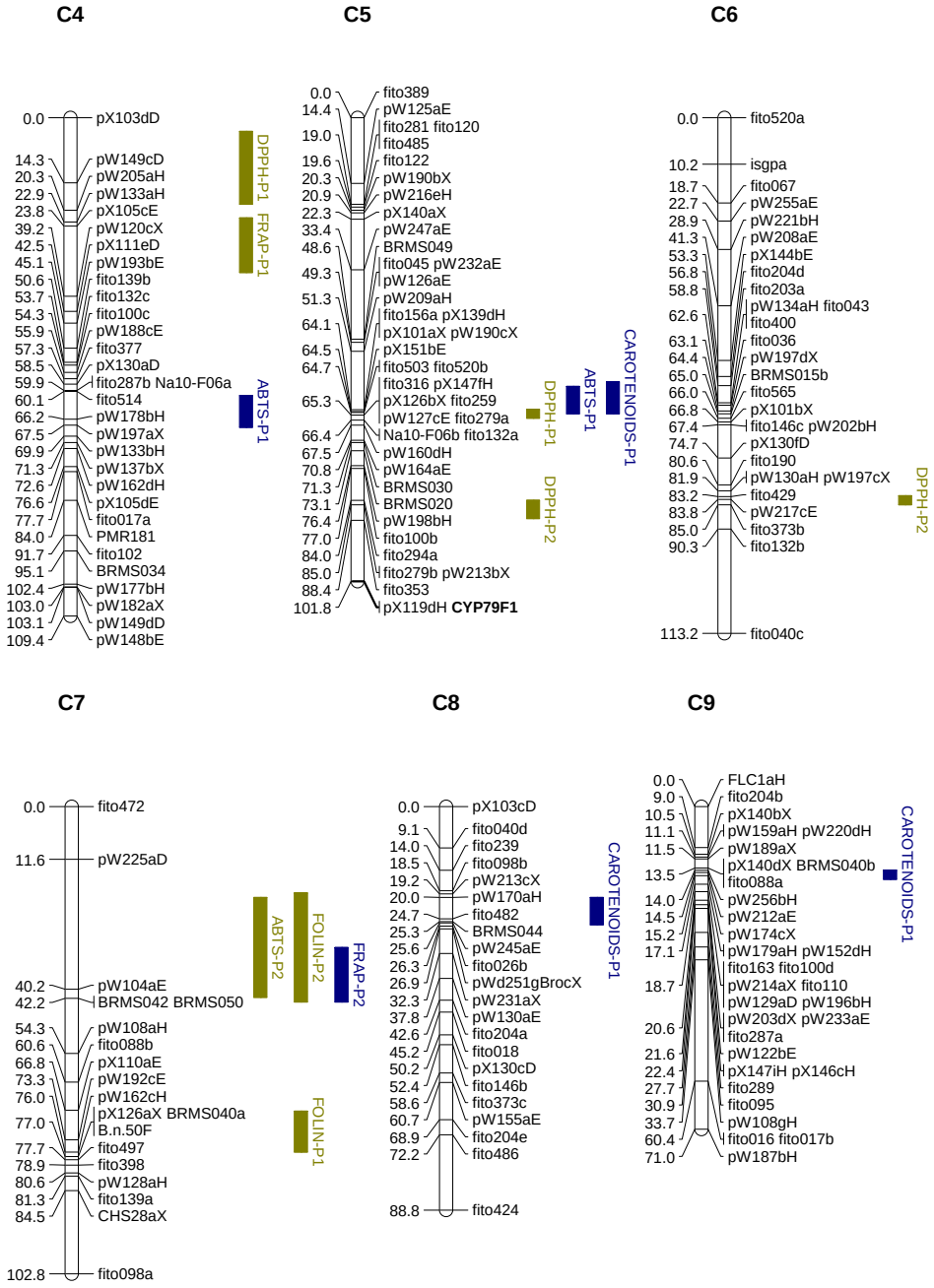


Figure 3.4: Framework map of DH population showing nineteen metabolic quantitative trait loci (QTL) for individual methods measuring AA. Linkage groups were labeled following the nomenclature of Iñiguez-Luy et al. (2009). Bars represent the LOD confidence interval of each QTL. QTLs are in different colors depending on the plant organ: leaves (green) and flower buds (blue). After the name of each QTL P1 indicates allele from, DH rapid cycling of Chinese kale (TO1000DH3) and P2 indicates allele from DH.



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Genes related to phenylpropanoid biosynthesis were located by means of *in silico* mapping in the confidence interval of several QTLs (Table 3.4). However no gene related to carotenoid biosynthesis could be located.

Table 3.3: List of quantitative trait loci (QTL) for antioxidant activity and the content of metabolites with antioxidant activity in two plant organs under study, leaves and flower buds.

	Plant organ	Trait	Linkage group	Peak Position range (cM)	Left marker	Right marker	Lod theshold	LOD score	Frequency	Add	R^2%	adj R^2%
1	**Leaves**	**ABTS**	7	31 (20-42)	pW225aD	pW104aE	2.89	4.53	829	1.6354	14.2	12.5
2	**Leaves**	**DPPH**	4	12 (3-19)	pX103dD	pW149cD	2.85	5.45	972	-1.794	17.4	27.2
3			5	65 (64-66)	fito316, pX147fH, pX126bX, fito259, pW127cE & fito279a	Na10-F06b & fito132a		4.5	764	-2.112	14.1	
4			5	85 (84-88)	fito294a			3.83	605	2.202	12.2	
5			6	84 (83-85)	pW217cE	fito279b & pW213bX		3.64	249	3.455	11.6	
6	**Leaves**	**PHENOLICS**	7	34 (19-43)	pW225aD	pW104aE	3.02	3.23	278	0.294	10.4	10.5
7			7	73 (67-76)	pX110aE	pW192cE		3.2	213	-0.268	10.3	

Additive effect was calculated as (P2-P1)/2; R^2 % coefficient of determination of each QTL. Adj R^2 %: determination coefficient of each trait.

Table 3.3: Continued

	Plant organ	Trait	Linkage group	Peak Position range (cM)	Left marker	Right marker	Lod theshold	LOD score	Frequency	Add	R^2%	adj R^2%
8	**Leaves**	**FRAP**	3	41 (32-46)	fito156c, pW133cH, pW166bH & pX128aX	pW125dE	2.86	3.05	299	2.784	9.8	11
9			4	25 (22-34)	pX105cE	pW120cX		4.29	794	-2.518	13.5	
10	**Flower buds**	**ABTS**	3	38 (31-44)	fito262	fito156c, pW133cH, pW166bH & pX128aX	2.86	2.98	260	1.329	9.8	6.6
11			4	64 (61-68)	fito514	pW178bH		3.49	501	-2.441	11.4	
12			5	64 (59-65)	pW209aH	fito156a, pX139dH, pX101aX & pW190cX		3.28	348	-1.141	10.7	

Additive effect was calculated as (P2-P1)/2; R^2 % coefficient of determination of each QTL. Adj R^2 %: determination coefficient of each trait.

Table 3.3: Continued

	Plant organ	Trait	Linkage group	Peak Position range (cM)	Left marker	Right marker	Lod theshold	LOD score	Frequency	Add	R^2%	adj R^2%
13	**Flower buds**	**DPPH**	3	45 (37-47)	fito156c, pW133cH, pW166bH & pX128aX	pW125dE	2.86	3.11	48	1.402	10.2	1.3
14	**Flower buds**	**FRAP**	3	12 (9-26)	pW212bE	fito272	2.83	3.38	462	-2.325	11	6.8
15			7	40 (31-43)	pW225aD	pW104aE		3.72	631	2.538	12.1	
16	**Flower buds**	**Anthocyanin**	3	72 (69-73)	fito227	pW196aH, fito488, pX131bX, pX119bH & pW219aE	3.12	3.26	361	-4.458	10.9	0.2

Additive effect was calculated as (P2-P1)/2; R^2 % coefficient of determination of each QTL. Adj R^2 %: determination coefficient of each trait.

Table 3.3: Continued

	Plant organ	Trait	Linkage group	Peak Position range (cM)	Left marker	Right marker	Lod theshold	LOD score	Frequency	Add	R^2%	adj R^2%
17	**Flower buds**	**Carotenoid**	5	64 (58-65)	pW209aH	fito156a, pX139dH, pX101aX & pW190cX	2.93	2.94	226	-0.044	9.9	21.2
18			8	22 (20-26)	pW170aH	fito482		3.27	308	-0.047	10.9	
19			9	15 (14-16)	pW212aE	pW174cX		3.8	583	-0.049	12.6	

Additive effect was calculated as (P2-P1) /2; R^2 % coefficient of determination of each QTL. Adj R^2 %: determination coefficient of each trait.

Table 3.4: List of phenylpropanoid biosynthesis candidate genes residing within the QTL confidence intervals according to organ and measurement method.

Plant organ	Trait	Markers in the confidence interval	Position in *Brassica oleracea* (cM)	*Brassica oleracea* linkage group	Linkage group and position (bp) in *Arabidopsis thaliana*	Genes related to phenylpropanoid biosynthesis located in the interval of *Arabidopsis thaliana*	Candidate genes
Leaves	**FRAP**	fito156c	38.72	3	1(3530200-3530221)		
		pW133cH	38.72	3	2 (15610858-15610982)		
		pX128aX	38.72	3	5 (6804683-6804766)		
		pW125dE	45.85	3	5(2219504-2219693)		
						AT5G48930	HCT
Flower buds	**ABTS**	fito250	36.12	3	5(15688217-26579698)		
						AT5G48930	HCT
Flower buds	**DPPH**	fito156c	38.72	3	1(3530200-3530221)		
		pW133cH	38.72	3	2 (15610858-15610982)		
		pX128aX	38.72	3	5 (6804683-6804766)		
		pW125dE	45.85	3	5(2219504-2219693)		
						AT5G48930	HCT

*Candidate gene found by means of *in silico* mapping in the *Arabidopsis thaliana* TAIR database. CHS and SS: Chalcone and stilbene synthase family protein; 4-CL: 4-coumarate: Co-A ligase 1, 2 or 3; HCT: hydroxycinnamoyltransferase enzyme; C4H: cinnamate 4-hydroxylase.

Table 3.4: Continued.

Plant organ	**Trait**	**Markers in the confidence interval**	**Position in *Brassica oleracea* (cM)**	***Brassica oleracea* linkage group**	**Linkage group and position (bp) in *Arabidopsis thaliana***	**Genes related to phenylpropanoid biosynthesis located in the interval of *Arabidopsis thaliana***	**Candidate genes**
Flower buds	**FRAP**	pW212bE	9.81	3	3(6427399-6427450)		
		fito066	26.12	3	4(6017387-6017408)		
						AT4G00040	CHS and SS
Leaves	**FRAP**	pX105cE	23.78	4	2(16117201-18117509)		
						AT2G40890	CYP98A3
Leaves	**DPPH**	pW217cE	83.82	6	1(14257280-18257453)		
						AT4G30210	
Leaves	**ABTS**	fito472	0	7	4(18268924-18269031)		
		pW104aE	40.25	7	1(126837519-26837557)		
						AT1G51680	4CL
Leaves	**phenolics**	fito472	0	7	4(18268924-18269031)		
		pW104aE	40.25	7	1(126837519-26837557)		
						AT1G51680	4CL
Flower buds	**FRAP**	fito472	0	7	4(18268924-18269031)		
		pW104aE	40.25	7	1(126837519-26837557)	AT1G51680	4CL

*Candidate gene found by means of *in silico* mapping in the *Arabidopsis thaliana* TAIR database. CHS and SS: Chalcone and stilbene synthase family protein; 4-CL: 4-coumarate: Co-A ligase 1, 2 or 3; HCT: hydroxycinnamoyltransferase enzyme; C4H: cinnamate 4-hydroxylase.

3.5. DISCUSSION

3.5.1. Quantitative variation for methods measuring AA and the content of metabolites with AA

Parents of the DH BolTBDH mapping population showed significant differences for the majority of the methods measuring AA and for the content of metabolites with AA in leaves and flower buds. BolTBDH population was found to be an ideal material in order to study QTLs for the traits under study in *Brassica* genus due to the differences between the two parents of this population. One parent (P2) is a broccoli 'Early Big' line, the brassica crop with one of the highest AA (Benzie et al., 2002), while the other parent (P1) is a DH rapid cycling line (TO1000DH3). Both parents are from different cultivars and as stated before, there is high variability for AA between different brassica crops (Podsedek et al., 2006; Samarth et al., 2008; Samec et al., 2011; Soengas et al., 2012).

The total AA of a sample can be measured by using several methodologies (Huang et al., 2005; Podsedek et al., 2006; Kusznierewicz et al., 2008). The radical scavenging capacity of DH BolTBDH mapping population was measured by using three ET methods: ABTS, FRAP and DPPH. The content of metabolites with AA like phenolics, anthocyanin and carotenoid was also measured. Some DH lines exhibited mean values of the traits falling between the values of the two parents, but others exhibited values which were extremely higher or lower than their parents. This phenomenon is referred to as transgressive segregation. Distributions of the methods measuring AA, phenolics and pigment content were, in most cases, transgressive. The action of complementary genes may be the primary cause of transgression, although epistasis may also contribute (Rieseberg et al., 1999). Further studies could help to explain the transgressive segregation of the traits measured in this study. These studies could use other populations or add more molecular markers to our population.

Total AA varied considerably according to the organ under study. Generally speaking, leaves present higher AA and content of metabolites with AA than flower buds, as it was expected by their photosynthetic complex. This result is in agreement with Soengas et al. (2012) and Llorach et al. (2003), who measured the AA of heads and leaves of cauliflower, with the highest values found in leaves. Guo et al. (2001) found

similar values in both organs in broccoli and Soengas et al. (2012) found that broccoli flower buds have higher AA than leaves. In broccoli and cauliflower, the organs which are consumed are the heads (flower buds) and the leaves surrounding the heads are treated as by-products. Our results show that leaves have more AA and content of metabolites with AA than heads. Therefore, consumption of broccoli by-products, which is one of the parents of the mapping population, could be an interesting option to include in the human diet.

Due to the characteristics of the methods analyzed, AA measured with FRAP and DPPH assays present lower values compared to that of ABTS assay. It is coincident with the results found by Gouveia et al. (2013) in other species like *Andryala glandulosa*.

3.5.2. Correlation coefficients among methods measuring AA and the content of metabolites with AA

Significant correlation coefficients were found among the three methods measuring AA (FRAP, DPPH and ABTS) in the two organs under study, and ranged between 0.19 and 0.53. These correlations, although significant, were lower than others found in previous studies. Kusznierewicz et al. (2008) found a correlation of 0.96 between ABTS and DPPH in white cabbage planted in different locations. Soengas et al. (2012) found a correlation of 0.8 between DPPH and FRAP in extracts of different brassica crops. Zhi-Xiang et al. (2011) found correlations ranging from 0.76 to 0.82 between the three cited methods analyzing different vegetables including broccoli. The material studied in our research is much closer genetically than the material studied in previously cited literature, since all the DH lines derive from a single cross. Clearly, correlations among ET methods depend on the material under study and based on our results, we recommend using more than one ET method in order to estimate the AA of a sample as suggested by Kusznierewicz et al. (2008) and Gawlik-Dziki (2008).

Significant correlations among the three methods measuring AA and the content of metabolites with AA were found in leaves and flower buds. Phenolic content was positively correlated with all the methods measuring AA. The correlation coefficient with ABTS showed the highest value in both organs. Several authors have found significant

and high correlations (ranging from 0.7 to 1) between the AA measured with ABTS, DPPH and FRAP assays and phenolic content measured with the Folin–Ciocalteu method in other brassica crops (cabbages, broccoli and Brussels sprouts) (Huang et al., 2005; Mrkic et al., 2006; Podsedek et al., 2006; Charanjit et al., 2007; Zhi-Xiang et al., 2011). These results confirm the hypothesis that phenolic compounds mainly account for the AA of brassica extracts. In the review made by Podsedek et al. (2006), it is pointed out that phenolic compounds have higher AA in *in vitro* experiments than vitamins and carotenoids.

Furthermore, positive and significant correlations between carotenoid content and methods measuring AA were found in flower buds (FRAP) and in leaves (ABTS) in this study. These correlations are smaller than those of phenolic compounds with AA. Our results confirm that carotenoids are metabolites which contribute to the AA of brassica extracts. Krinsky et al. (2001) described that phenolic and carotenoid content is positively correlated with AA. In the case of anthocyanins, our experiments do not show a clear relationship between their content and methods measuring AA.

3.5.3. QTL mapping for methods measuring AA and the content of metabolites with AA

Methods measuring AA on food extracts are extensively used by the scientific community in order to detect potential benefits for human health. Genetic variation for these traits is interesting from the breeder's points of view, since it could allow increasing the AA of brassica foods by selection. As far as we know, no report of QTLs or genetic basis for methods measuring AA has been done before in any brassica crop. This is also one of the first assays, which studies the genetic base of ET methods measuring AA in any crop. Only three recent pieces of research in rice (Jin et al., 2009), raspberry (Dobson et al., 2012) and in lettuce (Hayashi et al., 2012) studied QTLs for total water AA, total phenolic content, anthocyanin and carotenoid content. Knowledge derived from this study can be utilized in order to search for genes underlying these traits.

Ten out of 19 QTLs determine AA or the content of metabolites with AA in only one of the two organs, thus indicating that the regulation of genes underlying several

QTLs is organ-dependent. Seven QTLs determined variation for only one method measuring AA, thus indicating that the genetic basis regulation is partially dependent on the method. Genomic regions AA-C3, AA-C5 and AA-C7 determined variation for more than one ET method measuring AA. These genomic regions could be responsible for the significant correlations found between ET methods in this study.

The genomic region AA-C7 determines variation for methods measuring AA and phenolic compounds and the genomic region AA-C5 determines variation for methods measuring AA and carotenoid content. These finding supports the hypothesis that AA of the mapping population is related to phenolic compounds but also to carotenoid content. No QTLs related to methods measuring AA and anthocyanin content were found. Therefore, anthocyanins would not play a significant role in maintaining the AA of extracts in this population. The content of other compounds different from those under study could be responsible for the remaining QTLs, which control variation for methods measuring AA.

The core reactions of phenylpropanoid metabolism involve several steps catalyzed by three key enzymes: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-Coumarate: CoA ligase (4CL) (Hahlbrock, 1989). In *A. thaliana* there are 4CL different genes. This enzyme has a pivotal role in the biosynthesis of a plant's secondary compounds at the divergence point from general phenylpropanoid metabolism to several major branch pathways (Pietrowska-Borek et al., 2003; Xu et al., 2011). After *in silico* mapping analysis, 4CL-1 gene was located in the genomic region AA-C7 which controls AA measured as ABTS and FRAP and phenolic content. The hydroxycinnamoyltransferase enzyme (HCT) appears to be potentially implicated in the pathway both upstream and downstream of the 3-hydroxylation step and it is another key enzyme in phenylpropanoid biosynthesis. HCT enzyme catalyzes reactions both immediately preceding and following the insertion of the 3-hydroxyl group into the monolignol pathway (Hoffmann et al., 2002; Hoffmann et al., 2004; Shadle et al., 2007) realised by the CYP98A3 (C3'H). HCT gene from *A. thaliana* was located by means of *in silico* mapping in the genomic region AA-C3, which controls AA measured with the three ET methods. C3'H gene was located in the interval of pX105cE to pW120cX on chromosome 4 where a QTL for AA measured with FRAP method was found. More candidate genes related to phenylpropanoid biosynthesis, along all the linkage group,

were identified as it is the case of the chalcone and stilbene (CHS and SS) family protein which catalyzed the initial steps for flavonoid biosynthesis, route related with the phenylpropanoid biosynthesis (Schroder and Romeo, 2000). More work is necessary in order to validate and confirm candidate genes for the QTLs found in this study.

3.6. CONCLUSIONS

No reports on the genetic basis of AA, and the content of metabolites with AA like phenolic, anthocyanin and carotenoid content have been documented before in brassica crops. Results among methods measuring AA suggest that it is necessary to use more than one ET method in order to estimate AA, due to the fact that these methods present low significant correlations between them. Phenolic compounds and carotenoids are responsible for the AA of brassica extracts.

Three genomic regions determined variation for more than one ET method measuring AA. QTL analysis confirms that AA of the mapping population is related to phenolic compounds but also to carotenoid content. It should be pointed out that the experiments have been carried on in one environment and under controlled conditions of temperature and light. Once the existence of QTLs for the traits under study has been proved, new experiments are going to be carried on in different environments to test the stability of the QTLs and the influence of environmental conditions. Several candidate genes related to phenylpropanoid biosynthesis are proposed for the QTLs found. These QTLs and the possible candidate genes identified through syntenic analysis with *A. thaliana* are the first step to understand the genetic basis of AA in the *Brassica* genus.

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CAPÍTULO IV

IDENTIFICATION OF METABOLIC QTLs AND CANDIDATE GENES FOR GLUCOSINOLATE SYNTHESIS IN *BRASSICA OLERACEA* LEAVES, SEEDS AND FLOWER BUDS

4. IDENTIFICATION OF METABOLIC QTLS AND CANDIDATE GENES FOR GLUCOSINOLATE SYNTHESIS IN *BRASSICA OLERACEA* LEAVES, SEEDS AND FLOWER BUDS

Tamara Sotelo, Pilar Soengas, Pablo Velasco, Víctor M. Rodríguez, M. Elena Cartea

Group of Genetics, Breeding and Biochemistry of Brassicas, Department of Plant Genetics, Misión Biológica de Galicia, Spanish Council for Scientific Research (MBG-CSIC), Pontevedra, Spain.

4.1. ABSTRACT

Glucosinolates are major secondary metabolites found in the *Brassicaceae* family. These compounds play an essential role in plant defense against biotic and abiotic stresses, but more interestingly they have beneficial effects on human health. We performed a genetic analysis in order to identify the genome regions regulating glucosinolates biosynthesis in a DH mapping population of *Brassica oleracea*. In order to obtain a general overview of regulation in the whole plant, analyses were performed in the three major organs where glucosinolates are synthesized (leaves, seeds and flower buds). Eighty two significant QTLs were detected, which explained a broad range of variability in terms of individual and total glucosinolate (GSL) content. A meta-analysis rendered eighteen consensus QTLs. Thirteen of them regulated more than one GSL and its content. In spite of the considerable variability of glucosinolate content and profiles across the organ, some of these consensus QTLs were identified in more than one tissue. Consensus QTLs control the GSL content by interacting epistatically in complex networks. Based on *in silico* analysis within the *B. oleracea* genome along with synteny with *Arabidopsis*, we propose seven major candidate loci that regulate GSL biosynthesis in the *Brassicaceae* family. Three of these loci control the content of aliphatic GSL and four of them control the content of indolic glucosinolates. GSL-ALK plays a central role in determining aliphatic GSL variation directly and by interacting epistatically with other loci, thus suggesting its regulatory effect.

Keywords: GSL-PRO, GSL-ALK, GSL-OH, CYP81F2, ATR1, metabolic quantitative trait loci

4.2. INTRODUCTION

The *Brassica* genus includes six agricultural important species which are grown in many countries, and important oil, condiment and vegetable crops. *Brassica* vegetables like broccoli, cabbage, Chinese cabbage, turnip greens and leaf rape, among others, are consumed throughout the world. FAO Statistics (FAOStat 2011) show that the production of cauliflower, broccoli, kales and other crucifers was 8.2% of the total vegetable production of the world in 2011. The most consumed crop of this genus in Europe and the USA is *Brassica oleracea*. This species includes cabbages, kales, broccoli and cauliflower, among others.

Glucosinolates (GSLs) are the major class of secondary metabolites found in the *Brassicaceae* family, including the *Brassica* genus. The hydrolytic breakdown products of GSLs (especially isothiocyanates) have beneficial effects on human health, such as cytotoxic and apoptotic effects in damaged cells, thus preventing cancer in humans and reducing the risk for degenerative diseases (Forte et al., 2008; Van Horn et al., 2008; Virgili and Marino, 2008). They also enhance plant protection to abiotic and biotic stresses (Fahey et al., 2001). GSLs could exhibit certain adverse effects. For example, progoitrin can cause goiter in animals (Liu et al., 2012), which provoked the deliberate reduction of GSL levels in *B. napus* in the past. However, there is no evidence of any goitrogenic effect coming from *Brassica* consumption in humans (Mithen, 2001). Currently, efforts are concentrated on increasing the level of health promoting GSLs in brassica crops. For example Sarikamis et al. (2006) selected broccoli for higher levels of 3-methylsulphinylpropyl (GIB) and 4-methylsulphinylbutyl (GRA), which are the precursors of the isothiocyanates called iberin and sulforaphane, respectively. The beneficial effects of both isothiocyanates on human health are well known, having an influence on carcinogenesis during the initiation and promotion phases of cancer development (Cartea and Velasco, 2008). Knowledge on the genetics underlying the synthesis and accumulation of GSLs in brassica crops is an important tool for designing appropriate strategies in order to increase the content of those GSLs related to human health and plant protection.

GSLs are divided into three different classes according to the amino acid precursor in biosynthesis: (1) aliphatic GSLs derived from alanine (Ala), leucine (Leu), isoleucine

(Ileu), valine (Val), and methionine (Met); (2) aromatic GSLs derived from phenylalanine (Phe) and tyrosine (Tyr) and (3) indolic GSLs derived from tryptophan (Trp) (Zukalova and Vasak, 2002).

In *Arabidopsis thaliana* and brassica crops, most GSLs are synthesized from Met. GSL biosynthesis is a tripartite pathway involving three independent steps (Figure 4.1A): (i) side chain elongation of some precursor amino acids such as Met and Phe, by adding one or several methylene groups. Chain elongation is carried out by methylthioalkylmalate synthase enzymes (MAM). (ii) Development of the core structure, which includes several steps: aldoxime formation catalyzed by the CYP79 family of cytochromes P450; aldoxime oxidation by the CYP83 family; thiohydroximic acid formation by conjugation to an S donor and after C-S bond cleavage; desulfoGLS formation by S-glucosyltransferase (S-GT); and GSL formation by sulfotransferase. (iii) Secondary modification of the amino acid side chain which includes oxidation, hydroxylation, methoxylation, desaturation, sulfation, and glycosylation (Sorensen, 1988; Mikkelsen et al., 2002).

To date, major genes and transcription factors involved in the three steps of GSL biosynthesis have been identified and characterized in the model plant, *A. thaliana*. Based on *A. thaliana* homology, three loci were identified in *B. oleracea* and cloned (Li and Quiros, 2002; Li and Quiros, 2003; Gao et al., 2006): two loci responsible for the elongation of the side chain of aliphatic GSLs named BoGSL-ELONG and BoGSL-PRO (homologous to MAM-1 and MAM-2 genes, respectively of *Arabidopsis*) and one locus responsible for side the chain desaturation and production of an alkenyl GSL named BoGSL-ALK (homologous to AOP2 gene of *Arabidopsis*). Afterwards, these loci, plus genes BoCS-lyase, BoGS-OH and BoCYP79F1, were mapped (Gao et al., 2007). However, genes responsible for other steps of the metabolic pathway remain undiscovered. Identification of metabolic QTLs is essential for the understanding of the quantitative genetic control of secondary metabolites and it is an early step to identify the genes underlying trait variation. The high co-linearity between *A. thaliana* and *Brassica* species can be used in order to identify candidate genes underlying QTLs that affect GSL content.

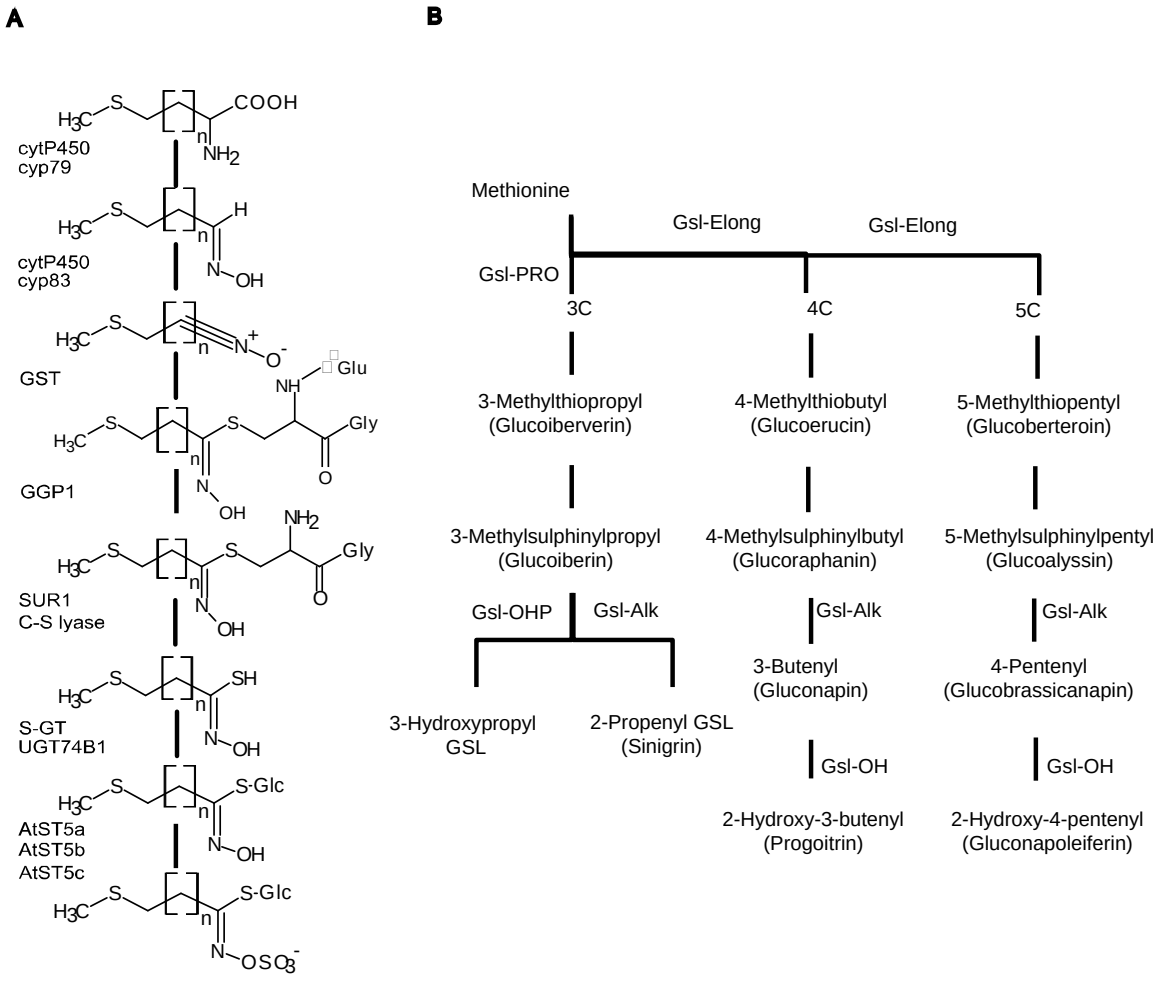


Figure 4.1: (A) Formation of the core structure of the three major groups of glucosinolates in *A. thaliana,* including the genes controlling this process (Mikkelsen et al., 2002; Feng et al., 2012). (B) A biochemical genetic model of the biosynthesis of aliphatic glucosinolates in *Brassicaceae* including the major genes controlling this process (Halkier and Du, 1997).

In addition to identifying structural and accumulation QTLs, it is important to determine the extent of epistatic interactions between loci which may play an important role in determining variability for GSL content.

The accumulation and profile of GSLs in plants are highly dependent on the genotype, although it is also affected by environmental and developmental factors. In *Arabidopsis*, GSL profiles have been systematically monitored during plant development and vary significantly among tissues and organs (Kliebenstein et al., 2001b; Petersen et al., 2002; Brown et al., 2003; Wentzell and Kliebenstein, 2008). In *B. oleracea*, developmental stages and the type of tissues may modify the type of GSLs and its levels (Velasco et al., 2007; Velasco et al., 2008). Currently, little is known about the genetics of GSL content within the plant ontogeny. For this reason, it is necessary to develop a better understanding of the genetics underlying GSL biosynthesis and accumulation in different tissues in *B. oleracea*.

In the present study we identify QTLs for GSL composition and accumulation in *B. oleracea* leaves, flower buds and seeds in a double haploid (DH) population. We also perform a comparative genomic analysis based on *A. thaliana-B. oleracea* synteny in order to find candidate genes underlying QTL variation. Epistatic relationships among QTLs are also described. This information may increase the understanding on the quantitative genetic control of these traits and it is useful in order to identify genes controlling GSLs in *B. oleracea*.

4.3. MATERIAL AND METHODS

4.3.1. Plant material and growing environments

A double haploid (DH) mapping population (BolTBDH) was employed in this work. The population was created from an F_1 individual, from a cross between a DH rapid cycling of Chinese kale (TO1000DH3, P_1) and a DH broccoli line 'Early Big' (P_2) (Iñiguez-Luy et al., 2009). TO1000DH3 is the reference genome for the *B. oleracea* sequencing project. Firstly, parents and 155 DH lines were grown and selfed in the greenhouse in 2010 under: 16 h of daylight and a temperature of 24 ± 2 °C; 8 h of darkness having 18 ± 2ºC at night; and a relative humidity of 55% in order to obtain enough seed in the same environmental conditions. Selfing was carried out by bagging each individual plant inside a microperforated polyethylene bags. Five bulks of 10 mg of seed for each line were prepared for GSL analysis with the seeds obtained. In 2011 (from September to

November), seeds from parents and 155 DH lines were sown with the same photoperiod and temperature as in 2010. Plants were sown in a completely randomized experiment with two replications and 4 plants per replication and DH line.

From each line, leave samples were taken at the 4 leaf stage and flower buds were taken differentially depending on the flowering time of each plant. One bulk was taken from each replication by mixing the four samples of leaves and flower buds. Samples were immediately frozen in liquid N_2, transferred to the laboratory and conserved at -80 °C until processing. All samples were lyophilized (BETA 2-8 LD plus, Christ) during 72 h. The dried material was powdered by using an IKA-A10 (IKA-Werke GmbH & Co.KG) mill, and the fine powder was used for GSL extraction.

4.3.2. GSL identification and quantification

Sample extraction and desulfation were performed according to Kliebenstein et al. (2001a) with minor modifications. Three microliters of the desulfo-GSL extract for seeds and 5 µl for leaves and flower buds were used in order to identify and quantify GSLs. Chromatographic analyses were carried out on an Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The UHPLC column was a C18 Atlantis® T3 waters column (3µm particle size, 2.1 x100 mm i.d.) protected with a C18 guard cartridge. The oven temperature was set at 30 ºC. Compounds were detected at 229 nm and were separated by using the following method in aqueous acetonitrile, with a flow of 0.8 mL min^{-1}: 1.5 minutes at 100% H_2O; a 11 min gradient from 0% to 25% (v/v) acetonitrile; 1.5 min at 25% (v/v) acetonitrile; a minute gradient from 25% to 0% (v/v) acetonitrile; and a final 3 min at 100% H_2O. Data were recorded on a computer with the LabSolutions software (Shimadzu). Specific GSLs were identified by comparing retention times with standards and by UV absorption spectra.

GSLs were quantified at 229 nm by using sinigrin (SIN, sinigrin monohydrate from Phytoplan, Diehm& Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS, glucobrassicin potassium salt monohydrate, from Phytoplan, Diehm& Neuberger GmbH, Heidelberg, Germany) as external standards and expressed in µmol g^{-1} dry weight

(DW). Calibration equations were made with, at least, five data points, from 0.34 to 1.7 nmol for sinigrin and from 0.28 to 1.4 nmol for glucobrassicin. The average regression equations for sinigrin and glucobrassicin were y = 148818x (R^2 = 0.99) and y = 263822 x (R^2= 0.99), respectively.

4.3.3. Statistical analysis

A combined analysis of variance across organs and individual analyses of variance for each organ were made for individual and total GSL. Lines and organs were considered as fixed factors and replications were considered as random factors. Analysis of variance was performed with the PROC GLM of SAS (SAS, 2011).

The genetic map employed for the QTL analysis was created by Iñiguez-Luy et al. (2009) having 279 markers (SSRs and RFLPs) distributed along nine linkage groups (C1-C9) with a total distance of 891.4 cM and a marker density of 3.2 cM/marker. Eight primer pairs described by Gao et al. (2007) amplifying loci BoGSL-ELONG, BoGSL-ALK, BoGSL-PROa, BoGSL-PRO-b, BoCS-lyase, BoGS-OH, BoCYP79F1 and BoS-GT from *B. oleracea* were screened in parent DH lines. Besides, SSRs Gi12 Hasan et al. (2008) and Ol12-D05 (Lowe et al., 2004) were screened in parental DH lines. SSRs Gi12 and Ol12-D05 map in both sides of ATR1 gene of *A. thaliana* in chromosome 5 (Hasan et al., 2008). Amplifications were performed by following Gao et al. (2007) and electrophoresis was carried out in 1% agarose gels and capillary electrophoresis system (CEQ 8000 Beckman, Coulter). Polymorphic markers were then screened in the BolTBDH mapping population, scored and assigned to linkage groups with JoinMap 3.0 sofware (Van Ooijen y Voorrips, 2001). The threshold for assigning markers to linkage groups was a LOD score between 5 and 8.

Quantitative trait locus mapping was carried out thanks to a composite interval mapping method (Zeng, 1994) by using the PLABQTL program (Utz and Melchinger, 2003). In each organ (leaves, flower buds and seeds), analyses were carried out on each individual GSL and for each GSL type (aliphatic, indolic and aromatic) as well as on the total GSLs. A likelihood odds (LOD) threshold of 3.2 was chosen in order to declare a putative QTL significant by following the method described by Van Ooijen (1999). The

confidence intervals were set at 95%. The analysis and cofactor election were carried out by following PLABQTL's recommendations, by using an 'F-to-enter' and an 'F-to-delete' value of 7.

The proportion of phenotypic variance explained for a specific trait was determined by the adjusted coefficient of determination of regression (R^2) fitting a model including all detected QTLs (Papst et al., 2004). Fivefold cross-validation of QTLs was performed by following the procedures described by Utz et al. (2000). The frequency of QTL detection gives us an estimation of the precision of QTL localization.

Significant QTLs for individual GSLs were integrated by using a QTL meta-analysis with BioMercator 2.1 software in order to give consensus QTLs (Goffinet and Gerber, 2000). An Akaike-type statistical criterion (AIC value) indicated the model which best fitted the data, including the number and the consensus QTLs positions. The aim of performing a meta-analysis was to find if a genomic region could determine the GSL content of different GSLs and if the same QTL was present in the three organs under study.

Iñiguez-Luy et al. (2009) identified collinear genomic blocks between the BolTBDH mapping population and *A. thaliana* by using a synteny analysis. This information was employed in order to identify candidate genes that may directly account for GSL QTLs in *B. oleracea.* In following this approach, we tried to locate 46 genes involved in GSL metabolism in *A. thaliana* which were obtained from TAIR (The *Arabidopsis* Information Resource) on the BolTBDH map by *in silico* mapping.

Epistatic interaction analysis among QTLs was performed by using the R/qtl package of the R software (Broman et al., 2003).

4.4. RESULTS

4.4.1. Phenotypic variation in GSL content

Twelve GSLs, belonging to three chemical classes, were detected in the BolTBDH population (Table 4.1). Eight GSL were aliphatic, three of them belonging to the 3C

group: 3-methylthiopropyl (GIV), 3-methylsulfinylpropyl (GIB) and 2-propenyl (SIN); four belonging to the 4C group: 4-methylthiobutyl (GER), 4-methylsulfinylbutyl (GRA), 3-butenyl (GNA) and 2-hydroxy-3-butenyl (PRO); and one belonging to the 5C group: 5-methylsulfinylpentyl (ALY). Three indolic GSLs: 4-hydroxy-3-indolylmethyl (OHGBS), 3-indolylmethyl (GBS); and 1-methoxy-3-indolylmethyl (NeoGBS), and one aromatic GSL, 2-phenylethyl (GNT), were also detected.

Different GSL profiles were detected in the parental lines (Figure 4.2). The following aliphatic GSLs were found in P_1 (TO1000DH3) in different organs: GIV, GIB, SIN GER, GRA, GNA, and PRO. Aliphatic GER and GRA and PRO were detected in P_2 ('Early Big' broccoli) meantime aliphatic ALY was found in the mapping population but it was not detected in its parents. Therefore, 3C and 4C GSLs were found in P_1, while only 4C GSLs were found in P_2. Alkenyl GSLs (SIN, GNA and PRO) were found in P_1 but not in P_2 (only trace amounts of PRO in flower buds) (Table 4.1).

The GSL profile of the mapping population varied depending on the organ. In leaves, 55.2% of GSLs were indolic and 40.2% of GSLs were aliphatic, being NeoGBS and GRA the major GSLs respectively. In seeds, 93.3% of total GSLs were aliphatic, and GRA, GNA and PRO were the major GSLs. The GSL profile of flower buds was intermediate among leaves and seeds as 67.7% of total GSLs were aliphatic and 28.6% were indolic. GRA, GNA and NeoGBS were the major GSLs in this organ. GIV and ALY were exclusively found in seeds, meanwhile GER was only found in flower buds and seeds (Table 4.1).

Aliphatic GSL content in P_1 was higher than that found in P_2 in the three organs analyzed (Table 4.1). SIN and GNA were the major aliphatic GSLs found in the three organs for P_1. In contrast GRA was the major GSL in P_2 in the three organs. Regarding indolic GSLs, GBS and NeoGBS were found as the most abundant in both parents in both leaves and flower buds, while OHGBS was the major GSL found in seeds. Indolic GSL content was higher in P_2 compared to P_1 in both leaves and flower buds.Total GSL content in P_1 was higher than that found in P_2 leaves and seeds (Table 4.1).

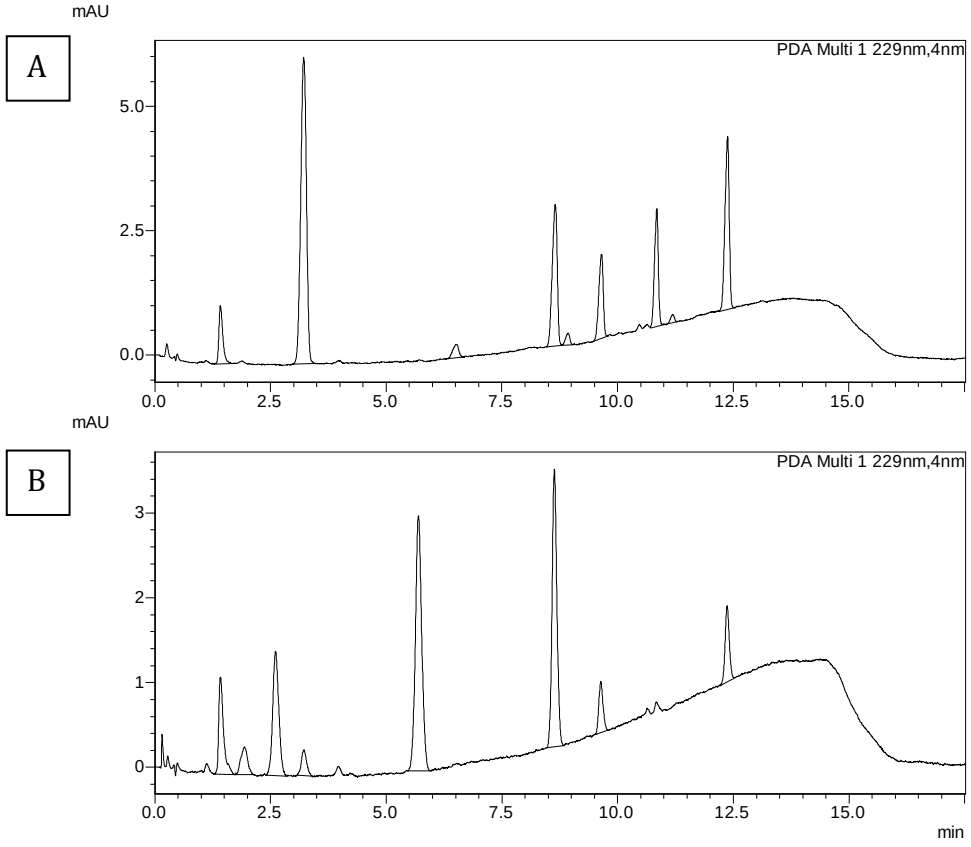


Figure 4.2: Chromatograms of glucosinolate (GSL) profiles of parents: TO1000DH3 (A) and Early Big (B).

In the mapping population, the content of individual GSLs as well as the content of aliphatic, indolic and total GSLs showed continuous distributions. Extreme phenotypes were found for all traits, with the exception of GNT in leaves, compared to phenotypes observed in parent lines (Table 4.1). For example, extreme mean values of some individual GSL content in the mapping population are far beyond the content of any of the parents. For instance, GRA content in seeds was 0.72 µmol g^{-1}dw in P_1 and 21.69µmol g^{-1}dw in P_2. The average GRA content in the mapping population was 22.62µmol g^{-1}dw and ranged from 0.48 to 74.14µmol g^{-1}dw (Table 1). Total GSL content in the different organs varied nearly 18-fold within the mapping population. The average content of total GSLs was 4.01 µmol g^{-1}dw in leaves,10.13 µmol g^{-1}dw in flower buds and 83.3 µmol g^{-1}dw in seeds (Table 4.1).

4.4.2. Analysis of variance

Significant organ × line interactions were found for all traits, therefore individual analyses were carried out by organ. The source of variation due to lines was highly significant for the most traits, except ALY and OHGBS in leaves and GIV and NeoGBS in seeds. The source of variation due to replications was in most cases nonsignificant (data not shown).

4.4.3. QTL analysis

Three out of eight primer pairs designed by Gao et al. (2007) were polymorphic in of the mapping population's parents. These markers could be mapped and located in three different linkage groups. BoGSL-OH mapped on C4 (28.8 cM), BoCYP79F1 mapped on C5 (102cM) and BoGSL-PROb mapped on C8 (66 cM). SSRs OL12-D05 and Gi12 were also polymorphic and they mapped on C8 (49 cM) and C9 (40 cM), respectively. QTL analyses were carried out with 279 markers designed by Iñiguez-Luy et al. (2009) and the five newly mapped primer pairs. No significant QTL was detected in any of the map positions where BoGSL-OH, BoCYP79F1 and BoGSL-PROb were located (Figure 4.3).

Eighty-two significant QTLs were detected being spread all over the 9 linkage groups of *B. oleracea*. The number of QTLs by linkage group ranged between two in C1 and 19 in C9 (Figure 4.3). Twenty significant QTLs were found in leaves. The value of R^2 ranged between 10.3% for GNA in C7 and 34.3% for the sum of aliphatic GSLs in C7 (Table S4.1). Half of QTLs had a frequency of cross-validation higher than 50%. Twenty-nine significant QTLs were detected in flower buds. R^2 value ranged between 10.4% for the sum of aliphatic GSLs in C3 and 49.7% for the sum of aliphatic GSLs in C9, respectively. Eighteen QTLs had a frequency of cross-validation higher than 50%. Thirty-three significant QTLs were found in seeds. R^2 value varied between 10.3% for the sum of indolic GSLs in C6 and 49.4% for ALY in C5. Twenty-eight QTLs had a frequency of cross-validation higher than 50%.

Table 4.1: Glucosinolate (GSL) profiles and concentrations (µmol g-1dw) of parents and mean and range of the DH population

	Leaves				Flower buds				Seeds			
GSL	**P_1**	**P_2**	**Population mean (range)**	**Population %**	**P_1**	**P_2**	**Population mean (range)**	**Population %**	**P_1**	**P_2**	**Population mean (range)**	**Population %**
GIV	-	-	-	-	-	-	-	-	0.53	0.00	1.63 (0-6.81)	1.39
GIB	0.00	0.00	0.29 (0-1.10)	5.33	0.00	0.00	0.89 (0-3.40)	6.60	1.04	0.00	6.06 (0-41.20)	5.14
SIN	2.42	0.00	0.44 (0-1.57)	8.02	1.57	0.00	1.22 (0-4.51)	9.04	42.32	0.00	8.15 (0-46.82)	6.91
GER	-	-	-	-	0.00	0.20	0.18 (0-0.50)	1.30	0.54	7.27	8.25 (0.27-34.54)	6.99
GRA	0.00	0.45	0.97 (0-6.65)	17.63	0.21	5.14	3.64 (0.15-17.35)	26.93	0.72	21.69	22.62 (0.48-74.14)	19.17
GNA	3.56	0.00	0.86 (0-6.38)	15.64	3.09	0.00	3.12 (0-17.12)	23.02	77.31	0.00	44.50 (0-138.40)	37.72
PRO	0.00	0.00	0.56 (0-2.77)	10.19	0.51	0.12	1.12 (0-13.22)	8.28	0.94	0.00	20.45 (0-129.80)	17.33
ALY	-	-	-	-	-	-	-	-	0.00	0.00	0.25 (0-2.38)	0.22
OHGBS	0.00	0.00	0.034 (0-0.36)	0.62	0.00	0.09	0.13 (0-0.41)	0.98	4.80	1.66	4.34 (1.81-10.20)	3.68
GBS	0.68	1.30	1.02 (0.005-3.24)	18.50	0.35	0.52	0.97 (0.14-3.87)	7.17	0.00	0.40	0.75 (0-5.37)	0.64
NeoGBS	1.72	2.34	1.14 (0.069-6.39)	20.63	0.59	1.06	1.86 (0.13-11.84)	13.78	0.53	0.37	0.50 (0-1.70)	0.43
GNT	0.19	0.79	0.19 (0-0.79)	3.44	0.18	0.86	0.39 (0-1.15)	2.90	0.38	0.21	0.42 (0-1.39)	0.36
Aliphatic	5.97	0.65	1.58 (0-6.97)	40.20	5.38	5.29	6.63 (0.59-20.98)	67.70	123.70	28.97	77.78 (30.38-157.15)	93.34
Indolic	2.40	3.65	2.17 (0.09-8.47)	55.21	0.94	1.68	2.88 (0.46-12.14)	28.57	5.33	2.44	5.29 (2.12-10.29)	6.36
Aromatic	0.19	0.79	0.19 (0-0.79)	4.22	0.18	0.86	0.39 (0-1.15)	3.86	0.38	0.21	0.42 (0-1.39)	0.50
Total	8.56	5.09	4.01 (0.12-13.20)	100.00	6.50	7.99	10.13 (1.47-24.56)	100.00	129.41	31.61	83.33 (36.23-160.29)	100.00

P_1, DH rapid cycling of Chinese kale (TO1000DH3); P_2DH broccoli line 'Early Big'; Aliphatic glucosinolates: GIV, Glucoiberverin; GIB, Glucoiberin; SIN, Sinigrin; GER, Glucoerucin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; ALY, Glucoalyssin; GBN, Glucobrassicanapin; Indolic glucosinolates: OHGBS, 4-hydroxyglucobrassicin; GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin; Aromatic glucosinolate: GNT, Gluconasturtiin.

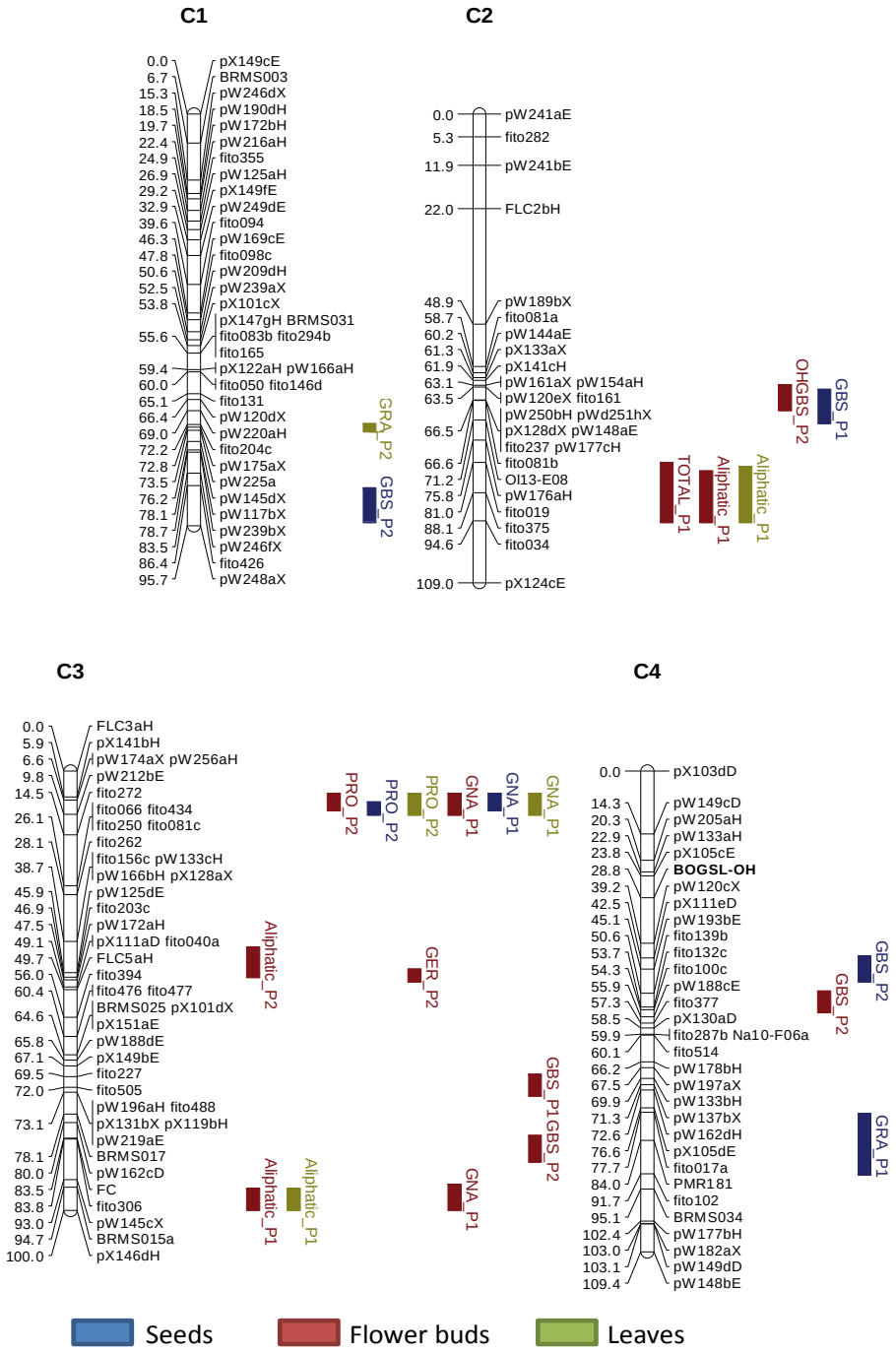


Figure 4.3: Framework map of DH population showing eighty-two metabolic quantitative trait loci (QTL) for individual GSLs and sums of GSLs. Linkage groups were labeled by following the nomenclature of Iñiguez-Luy et al. (2009). Bars represent the LOD confidence interval of each QTL. QTLs are in different colors depending on the plant organ: leaves (green), flower buds (red) and seeds (blue).

After the name of each QTL, -P_1 indicates allele from DH rapid cycling of Chinese kale (TO1000DH3) and -P_2 indicates allele from DH broccoli line 'Early Big'.

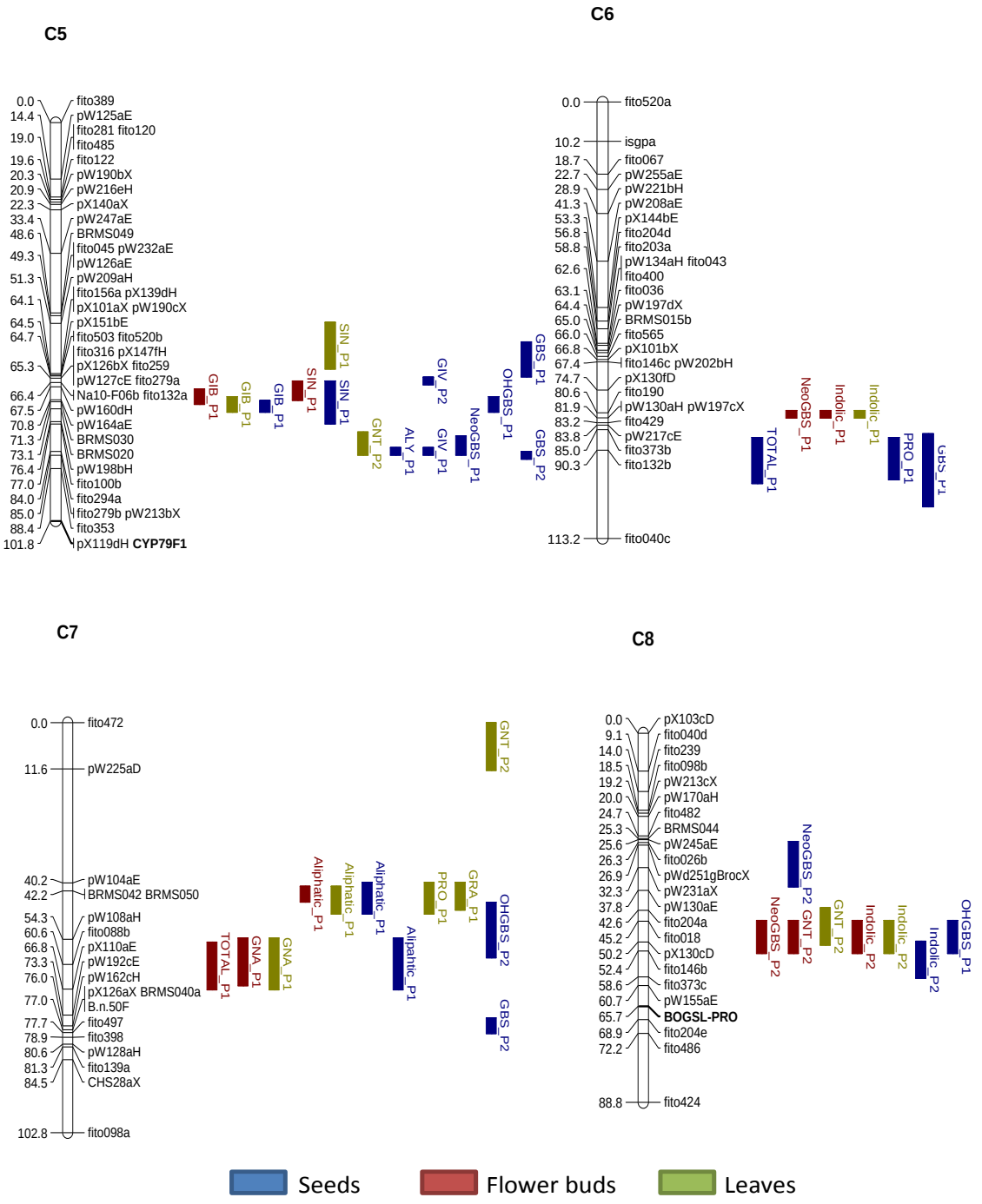
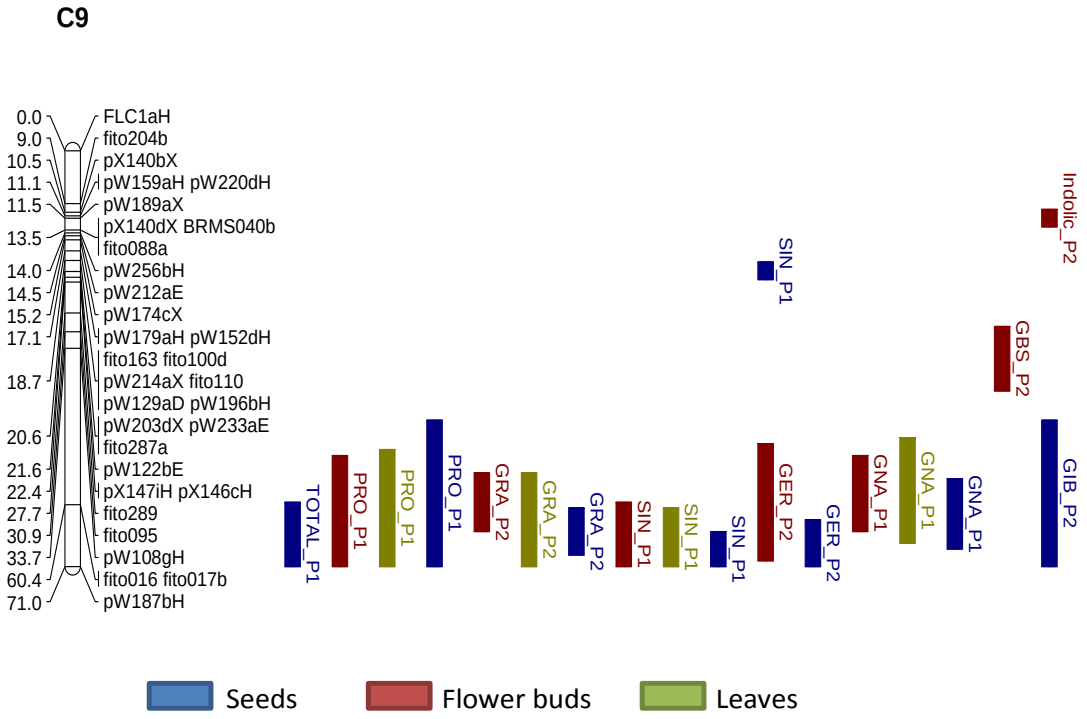


Figure 4.3: Framework map of DH population showing eighty-two metabolic quantitative trait loci (QTL) for individual GSLs and sums of GSLs. Linkage groups were labeled by following the nomenclature of Iñiguez-Luy et al. (2009). Bars represent the LOD confidence interval of each QTL. QTLs are in different colors depending on the plant organ: leaves (green), flower buds (red) and seeds (blue). After the name of each QTL, -P_1 indicates allele from DH rapid cycling of Chinese kale (TO1000DH3) and -P_2 indicates allele from DH broccoli line 'Early Big'.



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4.4.4. Consensus QTLs

Based on the position of the QTLs and taking into account their confidence interval, a meta-analysis in order to render consensus QTLs for GSL concentration was carried out. Eighteen consensus QTLs were detected (Table 4.2). Fourteen consensus QTLs were present in seeds, 12 QTLs in leaves and 14 QTLs in flower buds. Seven QTLs were common to flower buds, leaves and seeds; three QTLs were exclusively found in leaves, two QTLs were exclusively found in flower buds and other two QTLs were exclusive

found in seeds. In order to make the discussion clearer, results regarding consensus QTLs are going to be presented according to each chemical GSL class.

4.4.5. Aliphatic GSLs

Located in C3, consensus QTL-3.1 controls the content of PRO and GNA in the three organs (Table 4.2). Alleles for increasing PRO content are given by P_1, while alleles for increasing GNA content are given by P_2 (Figure 4.3). Consensus QTL-5.1, located in C5, controls the content of GIB and SIN in the three organs. Alleles for increasing the content of both GSLs are given by P_1. In C9, consensus QTL-9.2, which controls the content of PRO, GNA, GRA, GER (4C-GSL) and SIN, and GIB (3C-GSL) in the three organs, was located. Alleles for synthesis of PRO, SIN and GNA are given by P_1, while alleles for increasing the content of GRA, GER and GIB are given by P_2 (Figure 4.3). Other QTLs which control aliphatic GSL content exclusively are QTL-1.1, QTL-2.2, QTL-3.1, QTL-3.2, QTL-3.4, QTL-4.2 and QTL-7.2.

4.4.6. Indolic and aromatic GSLs

Several consensus QTLs only controlled the indolic GSL content. QTL-1.2, QTL-3.3, QTL-4.1 and QTL-7.4 determined the GBS content in seeds and flower buds (Table 4.2). Alleles for increasing the content of GBS are given by P_2 in all these QTLs except for QTL-3.3, where alleles came from both parents. Consensus QTL-2.1 determines the content of OHGBS and GBS in seeds and flower buds. The allele for increasing OHGBS is given by P_2 in flower buds, while the allele for increasing GBS content is given by P_1. Consensus QTL-8.1 determines the OHGBS, NeoGBS and total indolic GSL content in the three organs. Besides, this QTL also controls the content of the aromatic GNT. Other QTLs for GNT content are QTL-5.2 and QTL-7.1.

Table 4.2: Position and characteristics of consensus QTLs found in BolTBDH mapping population.

				Aliphatic																	
				3C			4C			5C				Indolic				Aromatic	Total	Plant organ		
LG	No	Peak position (cM)	Confidence interval (cM)	GIV	GIB	SIN	GER	GRA	GNA	PRO	ALY	GBN	Sum of aliphatic GSLs	OHGBS	NeoGBS	GBS	Sum of indolic GSLs	GNT	Total GSL	Seeds	Leaves	Flower buds
2	2.1	67.55	62.3-78.9											x		x				x		x
	2.2	89.74	83.7-95.7										x						x		x	x
3	3.1	7.09	4.9-9.3						x	x										x	x	x
	3.2	46.6	36.4-56.8				x						x									x
	3.3	79.1	69.0-89.2													x						x
	3.4	99.3	92.6-105.9						x				x								x	x
4	4.1	47.3	37.1-57.4													x				x		x
	4.2	84.0	70.9-97.0					x												x		
5	5.1	70.2	68.3-72.0	x	x	x								x		x				x	x	x
	5.2	84.1	81.9-86.3	x							x				x	x		x		x	x	
6	6.1	86.8	81.9-91.7							x					x	x	x		x	x	x	x
7	7.1	2.0	0-17.8															x			x	
	7.2	42.9	40.3-45.4					x		x			x							x	x	x
	7.3	56.4	52.3-60.5						x				x	x					x	x	x	x
	7.4	76.0	67.7-84.2													x				x		
8	8.1	45.8	41.5-50.1											x	x		x	x		x	x	x
9	9.1	27.0	20.7-33.3			x										x	x			x		x
	9.2	64.9	63.2-66.6		x	x	x	x	x	x									x	x	x	x

Aliphatic glucosinolates: GIV, Glucoiberverin; GIB, Glucoiberin; SIN, Sinigrin; GER, Glucoerucin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; ALY, Glucoalyssin; GBN, Glucobrassicanapin. Indolic glucosinolates: OHGBS, 4-hydroxyglucobrassicin; GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin. Aromatic glucosinolate: GNT, Gluconasturtiin.

The genomic regions QTL-1.2, QTL-2.2 and QTL-7.4 are collinear with genomic regions of *A. thaliana* in chromosomes 4, 5 and 2. In these regions, genes CYP83B1, CYP81F2 and CYP79B3 from *A. thaliana* were found by means of *in silico* mapping.

4.4.7. Epistatic networks

A total of 85 significant epistatic interactions were found when taking into account the three organs and all the traits. Thirteen epistatic interactions were found in leaves, 52 in flower buds and 13 in seeds. Some of these interactions are common to the three organs under study. Sixty-eight interactions were detected in aliphatic GSLs, 13 in indolic GSLs and 4 in total GSLs. An average of 3.5 significant epistatic interactions was found per trait (Figure S4.1).

Forty-two interactions were detected between QTLs, being two of them negative. Twenty interactions were detected between QTL-9.2 (proposed as GSL-ALK in this work) and other QTLs in traits related to aliphatic GSLs (Figure 4.4). The relationship between QTL-9.2 and QTL-3.1 (proposed as GSL-OH) was found for the aliphatic GNA, PRO, GER and GIB in the three organs under study. The relationship between QTL-9.2 and QTL-5.1 (proposed as GSL-PRO) was found for the aliphatic GER, SIN and GNA in the three organs (Figure 4.4). In the network controlled by GSL-ALK, interactions between aliphatic and indolic QTLs were observed. For example, QTLs-3.3, 4.1 and 9.1 control the GBS content and the three of them interact with QTL-9.2 in order to produce aliphatic GSLs (Figure 4.4).

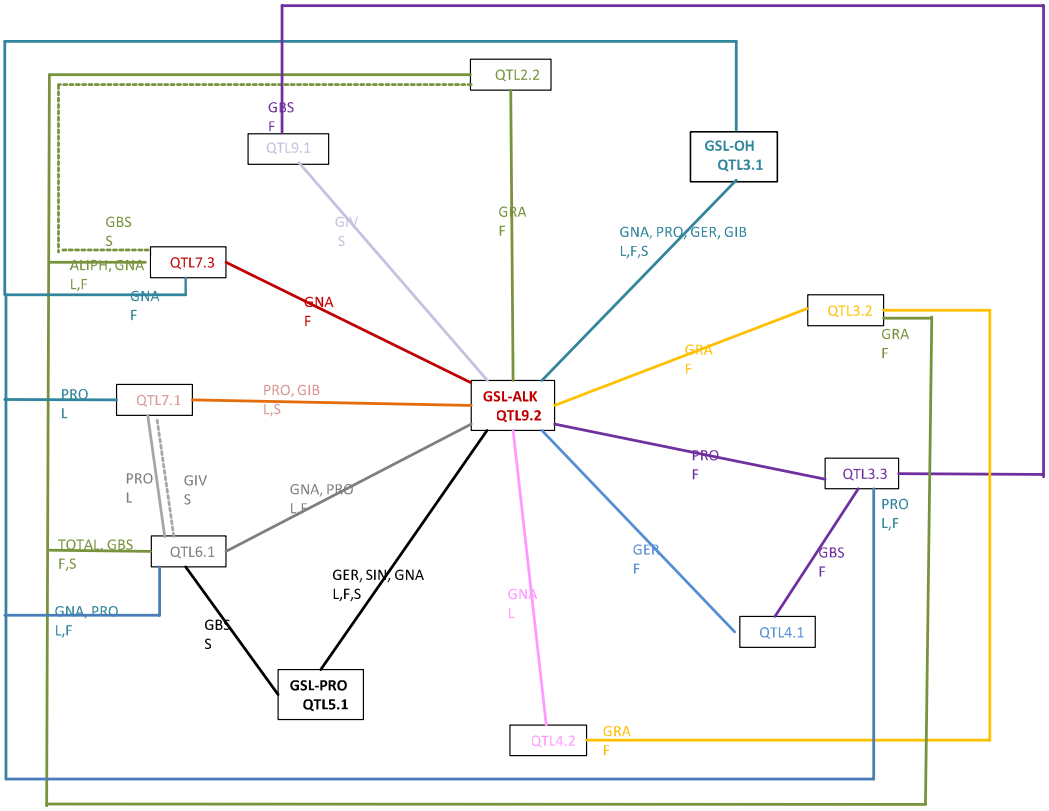


Figure 4.4: An epistatic network including all the significant relationships of QTL9.2 (GSL-ALK) with other QTLs. Aliphatic glucosinolates: GIV, Glucoiberverin; GIB, Glucoiberin; SIN, Sinigrin; GER, Glucoerucin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; ALY, Glucoalyssin; GBN, Glucobrassicanapin; ALIPH: sum of aliphatic GSLs; Indolic glucosinolate: GBS, Glucobrassicin; TOTAL: sum of total GSLs. Organs: L, Leaves; F: Flower buds; S: seeds. Continuous lines represent positive epistatic interactions while dashed lines represent negative epistatic interactions.

4.5. DISCUSSION

4.5.1. Phenotypic variation in GSL content

Parents of the mapping population had different GSL profiles and concentration. Particularly, parent P_2 has a higher concentration of GRA and a lower concentration of GNA than parent P_1 in the three organs. GRA is found in several *B. oleracea* crops like

cauliflower, cabbage and kale, although high levels of GRA equivalent to those found in P_2 ('Early Big' broccoli) are always found in broccoli (Li et al., 2001b; Verkerk et al., 2009; Bjorkman et al., 2011; Wang et al., 2012). The effect of sulforaphane, the isothiocyanate derived from GRA, against cancer has been reviewed in detail (Fahey et al., 1997; Cartea and Velasco, 2008). As a result of these epidemiological and biomedical studies, GRA is now viewed as a quality trait in *B. oleracea* crops to be targeted in breeding programs.

Distributions of individual and sums of GSLs were in most cases transgressive. These types of segregations have been described before for GSL content in *Brassica* (Mahmood et al., 2003; Ramchiary et al., 2007) and could be due to new combinations of additive alleles or to epistatic interactions among loci for GSLs, which have already been described (Kliebenstein et al., 2001; Feng et al., 2012).

Total GSL content varied considerably depending on the organ under study. As it was expected, seeds accumulated the highest GSL content followed by flower buds and leaves. After studying the GSL content in different organs of *A. thaliana*, Brown et al. (2003) found that seeds had the highest concentration followed by inflorescences, siliques, leaves and roots. Velasco et al. (2007) found that the GSL content in flower buds was higher than kale leaves. These results may reflect the need to indicate *de novo* synthesis of GSLs and/or mobilization (Brown et al., 2003).

The GSL profile also varied considerably depending on the organ. In fact, seeds were mostly composed of aliphatic GSLs, whereas indolic GSL were predominant in leaves. Flower buds had an intermediate profile. Besides, flower buds and seeds showed more diversification of aliphatic GSLs, since GIV and ALY were only found in seeds and GER was only found in flower buds and seeds. Agreeing with these results, kale leaves are characterized by high amounts of indolic GSLs during the first plant stages, while aliphatic GSLs are predominant in flower buds and in leaves taken at the end of the vegetative stage (Velasco et al., 2007). A similar pattern was observed in *A. thaliana*, where seeds are distinguished by unique aliphatic constituents and low level of indolic compounds. After germination, the proportion of aliphatic GSLs declined with age, thus resulting in the predominance of indolic GSLs by the time of senescence (Brown et al., 2003).

4.5.2. QTLs analysis

Seven out of 20 consensus QTLs determined the content exclusively in one of the three organs under study. Our results suggest that the regulation of genes underlying several QTLs is organ-dependent. Feng et al. (2012) analysed QTLs for GSL content in leaves and seeds of *B. napus* and found 17 QTLs which were exclusively detected in leaves. Kliebenstein et al. (2001) found three organ-specific QTLs for aliphatic GSLs in both leaves and seeds of *A. thaliana.* A similar number was found for indolic GSLs.

4.5.3. Aliphatic GSLs

Several major loci determine the profile and content of aliphatic GSLs in *Brassica* (Kliebenstein, 2009). The GSL-ELONG and GSL-PRO loci regulate the side chain length (Figure 4.1B). The presence of 4C-GSL is controlled by a dominant allele of GSL-ELONG (GSL-ELONG+), whereas the presence of 3C-GSL is controlled by a dominant allele of GSL-PRO (GSL-PRO+) (Li et al., 2001a). GSL-ALK controls side chain desaturation. The presence of GSL-ALK+ in 3C-GSL determines the production of alkenyl GSL. GSL-OHP catalyzes production of 2-hydroxypropyl GSL, but this GSL was not detected in parents or the mapping population. GSL-OH controls PRO production and its action is conditioned by the presence of GSL-ALK+ (Li et al., 2001a). After analyzing parents of the mapping populations, it can be concluded that the genotype of P_1 is GSL-ELONG+, GSL-PRO+, GSL-ALK+ and GSL-OH+, while the genotype of P_2 is GSL-ELONG+, GSL-PRO-, GSL-ALK-. Because P_2 is GSL-ALK- and the presence of GSL-ALK+ is needed in order to produce hydroxylated GSL, the genotype for the locus GSL-OH could not be determined. GSL-ELONG cannot be located into the mapping population, because both parents had the same genotype for this locus. Primer pairs amplifying loci GSL-PROb and GSL-OH designed by Gao et al. (2007) were located in the mapping population in different positions as those reported by the authors, thus probably indicating an unspecific amplification of PCR products.

Consensus QTL-5.1 controls the amount of three 3C-GSLs: GIB, GIV and SIN. Alleles for increasing 3C-GSLs content are given by P_1. Thus, GSL-PRO would be a good candidate gene for this QTL. This major locus was cloned (Gao et al., 2006) and

mapped at the top of C5 in *B. oleracea* (Gao et al., 2007). Position of C5 markers in the map of Iñiguez-Luy et al. (2009) is inverted with regard to C5 in the map of Gao et al. (2007). Taking this into account, the position of QTL-5.1 coincides with that of GSL-PRO. This information together supports the validation of the candidate gene. This QTL also controls the content of two indolic GSLs GBS and NeoGBS. Aliphatic and indolic GSLs are synthesized and subsequently modified by two independent parallel pathways (Wentzell et al., 2007). However, there are cross-talks between both pathways. Wentzell et al. (2007) found that GSL.INDOLIC.IV.8 and GSL.INDOLIC.V.20 QTLs, which control the content of several indolic GSLs in *A. thaliana*, map in the same genomic locations as GSL-AOP and GSL-ELONG loci which control aliphatic GSLs (Wentzell et al., 2007).

Consensus QTL-9.2 controls the amount of several GSLs. Alleles for increasing alkenyl GSL content (SIN, PRO, GNA) are given by P_1, while alleles for increasing non alkenyl GSL content (GRA, GER, GIB) are given by P_2 (Figure 4.1B). Locus GSL-ALK was studied and cloned by Li and Quiros (2003) and mapped in C9 (Gao et al., 2007) in the same position as QTL-9.2. Consensus QTL-3.1 controls the amount of GNA and its hydroxylated form PRO (Figure 4.1B). Curiously, alleles for increasing GNA content are given by P_1which is GSL-OH+, while alleles for increasing PRO content are given by P_2. This makes us think that P_2 is also GSL-OH+. The function of this QTL would correspond to gene GSL-OH. Gao et al. (2007) mapped this gene in C9, close to GSL-ALK. The position of the gene does not correspond to QTL-3.1. After searching in the whole genome sequence of *B. rapa*, Zang et al. (2009) and Wang et al. (2011) found GSL genes homologous to those of *A. thaliana*. Three different copies of gene GSL-OH were found in *B. rapa* due to the triplicate nature of its genome (Wang et al., 2011). Several copies of the same genes could also exist in *B. oleracea*.

During the first stage of the development of the core structure of aliphatic GSL (Figure 4.1), the gene CYP79F1 metabolizes mono- to hexahomomethionine into their corresponding aldoxime in *A. thaliana* (Chen et al., 2003). Primers designed in order to amplify this gene in *B. oleracea* (Gao et al., 2007) were employed in this work. CYP79F1 mapped in C5, in the same position found by Gao et al. (2007), but no QTL was found in this position, thus indicating that both parents have the same allele for this gene. Consensus QTL-2.2 controls the content of total aliphatic GSLs in leaves and flower buds

and the total GSL content in flower buds, but it does not control the content of any individual GSL, thus suggesting that the gene underlying this QTL may have a regulatory role in the aliphatic GSL pathway. Two R2R3-Myb transcription factors (Myb 28 and Myb 29) positively control biosynthesis of aliphatic GSLs in *A. thaliana* (Hirai et al., 2007) and could be candidate genes for this consensus QTL.

4.5.4. Indolic and aromatic GSLs

In the first stage of the development of the core structure (Figure 4.1A) of indolic GSLs, two cytochromes P450 (CYP79B2 and CYP79B3) catalyze the conversion of Trp to indole-3-acetaldoxime in *A. thaliana* (Hull et al., 2000; Mikkelsen et al., 2000). Overexpression of CYP79B2 results in an increased accumulation of indole GSLs, specifically 3-indolylmethyl (GBS) and 4-methoxy-glucobrassicin (MeOH-GBS) (not detected in this work). In the next step, CYP83B1 catalyzes the transformation of indole-3-acetaldoxime into to *S* -alkyl-thiohydroximate (Figure 4.1A) (Bak et al., 2001; Naur et al., 2003). The Myb transcription factor ATR1 from *A. thaliana* regulates the expression of genes CYP79B2, CYP79B3, and CYP83B1. Overexpression of ATR1 leads to lines with higher levels of total indolic GSLs than wild-type plants (Celenza et al., 2005). CYP81F2 catalyzes the hydroxylation at position 4 of the indole ring of GBS, which results in the formation of OHGBS and MeOH-GBS (Pfalz et al., 2009).

After *in silico* mapping of *A. thaliana* GSL genes, CYP79B2 and CYP79B3 were located inside the confidence interval of consensus QTL-1.2 and QTL-7.4. Both of them determine variation for GBS in seeds, agreeing with a possible high expression of candidate genes CYP79B2 and CYP79B3.

SSRs Gi12 and Ol12-D05 map in both sides of ATR1 gene of *A. thaliana* in chromosome 5 (Hasan et al., 2008). Gi12 mapped in C9 in our work, where no QTL was detected. Ol12-D05 mapped within the consensus QTL-8.1 confidence interval. This QTL determines variation for OHGBS, NeoGBS and total indolic GSL content in the three organs analyzed.

The high apparition of QTLs for indolic GSL content agrees with a high expression of ATR1 candidate gene. Besides, aromatic GNT is also controlled by this QTL. Aromatic GSLs are also a substrate of CYP83B1, regulated by ATR1. These results together suggest that ATR1 could be a possible candidate gene for QTL-8.1.

Consensus QTL-2.1 determines variation for OHGBS and GBS in flower buds and seeds. Candidate gene CYP81F2, metabolizing the step from GBS to OHGBS from *A. thaliana*, was found in the confidence interval of this QTL.

The *B. oleracea* whole genome sequencing is currently carried out by using TO1000DH3 as the reference genome. Sequences are being aligned by using mapping population BolTBDH. *B. oleracea* sequencing project will be a great opportunity to link sequences with the QTLs described in this work.

4.5.5. Epistatic networks

Significant epistatic interactions were found for the three organs under study. On the contrary of what was found by Feng et al. (2012) in *B. napus*, part of the interactions were common among organs. The number of interactions was higher in flower buds, thus indicating a more complex regulation of GSL biosynthesis in this organ. Epistatic interactions for indolic GSLs were less complex than for aliphatic GSLs. 49% of the epistatic interactions detected were between QTLs, thus indicating that variability for GSLs content is determined directly by QTLs and indirectly by interacting with other loci.

Epistatic interactions among GSL-ALK, GSL-PRO and GSL-OH, determine variability for aliphatic GSL content and have been described before (reviewed by Kliebenstein (2009)) in *A. thaliana*. They are mediated by transcriptional factors. In this work we have found that GSL-ALK plays a central role in the network of epistatic interactions for aliphatic GSLs, suggesting a possible regulatory effect of this locus. Indirectly, GSL-ALK also controls the variability for the indolic GSL named GBS, thus indicating cross-talk between indolic and aliphatic pathways. This information supports the results found by Wentzell et al. (2007) in *A. thaliana*. These authors transformed a null accession for AOP2 and AOP3 genes (GSL-ALK locus) with AOP2 gene from *B.*

oleracea, thus resulting in the production of alkenyl GSLs, doubling of total aliphatic GSL content and the induction of aliphatic GSL biosynthetic genes and regulatory genes.

4.6. CONCLUSIONS

An extensive analysis of QTLs controlling GSL variation in three different organs of *B. oleracea* has been presented. Possible candidate genes for different QTLs have been proposed based on the phenotypic study of the progeny and on the synteny with *A. thaliana*. Epistatic interactions among QTLs have been detected showing a central role of GSL-ALK in determining aliphatic GSL variation and suggesting a regulatory effect of this locus. Further work is going to be carried out in order to validate them and to find new candidate genes for remaining QTLs.

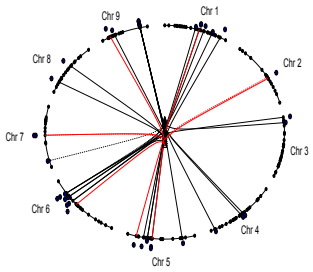
4.7. SUPORTING INFORMATION

Figure S4.1

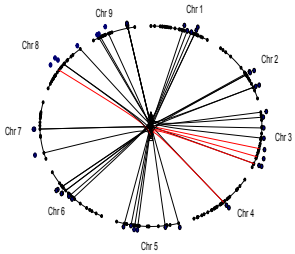
(a) Complex epistatic interactions in seeds, flower buds and leaves of *Brassica oleracea*. (b) Epistasis network for all analysed glucosinolates. Red lines indicate epistatic interactions for indolic glucosinolates and black lines for aliphatic glucosinolates. Epistasis network for individual glucosinolates. In both panels, dot and solid lines indicate negative and positive epistasis, respectively.

(a)

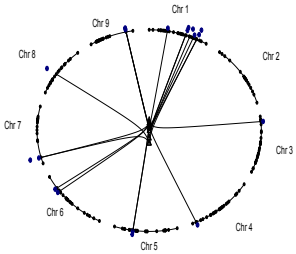
Seed



Flower bud



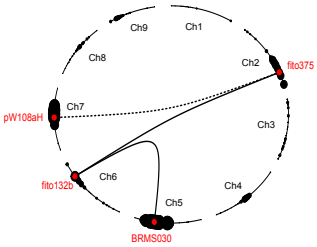
Leaf



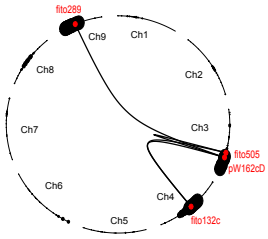
(b)

Seed

GBS

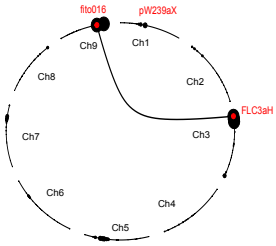


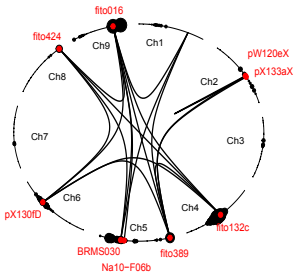
Flower bud



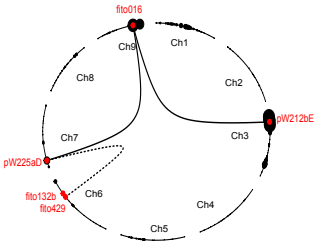
Leaf

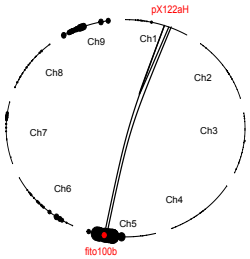
GER



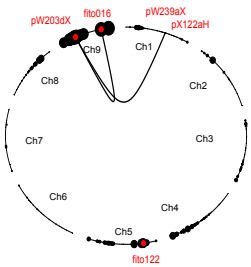


GIB





GIV



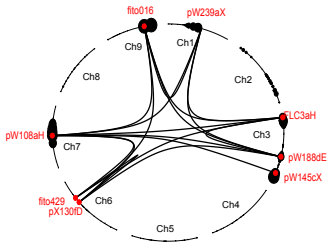
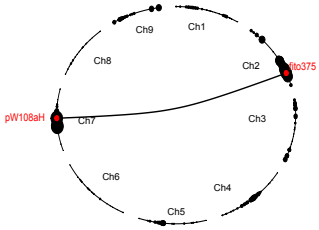
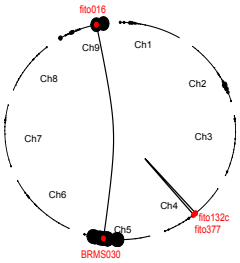
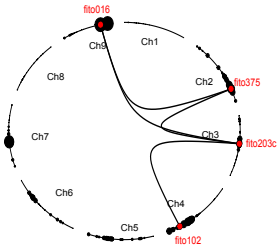
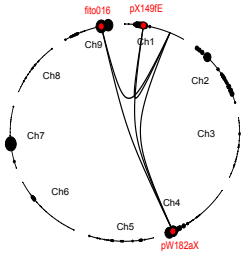
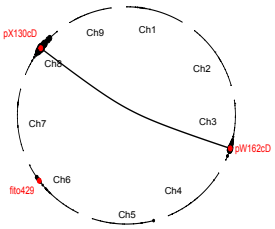
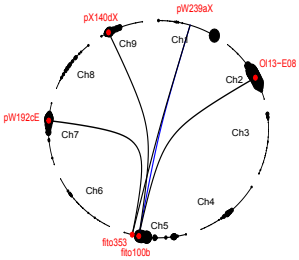
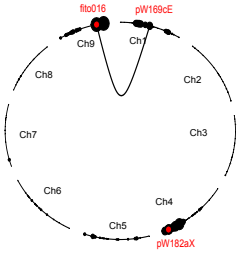
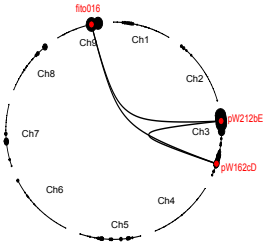
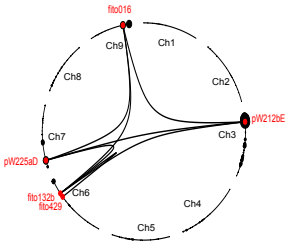
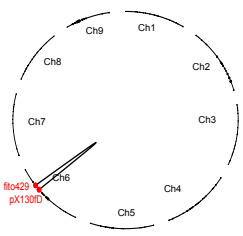
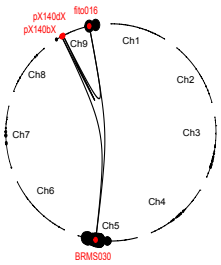
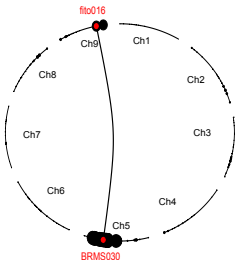
	Seed	Flower bud	Leaf
GNA			
GRA			
NeoGBS			
OHGBS			
PRO			
SIN			

Table S4.1: List of metabolic quantitative trait loci (QTL) for glucosinolatos (GSLs) in the three plant organs.

Plant organ	Trait	Linkage group	Peak Position	Confidence interval (cM)	Left mark	Right mark	LOD	Cross validation frequency (%)	Additive effect	R^2%	Adj R^2%
Leaves	GIB	5	72	70-74	BRMS030	BRMS020	8.5	98.0	-0.108	25.2	21
	SIN	5	57	51-63	pW209aH	fito156a	6.76	99.9	-0.119	20.6	20.6
		9	71	61-71	fito017b	-	3.68	39.0	-0.077	11.8	
	GRA	1	73	72-74	pW175aX	pW145dX	4.82	63.1	0.564	15.4	24
		7	42	40-47	pW104aE	pW108aH	5.6	30.5	-0.611	17.6	
		9	65	55-71	fito017b	pW187bH	5.01	99.3	0.461	15.9	
	GNA	3	7	5-10	pW174aX & pW256aH	pW212bE	3.91	45.2	-0.284	11.0	25.5
		7	55	54-67	pW108aH	fito088b	3.64	35.5	-0.268	10.3	
		9	62	49-67	fito016 & fito017b	pW187bH	9.17	100.0	-0.413	23.9	
	PRO	3	7	5-10	pW256aH	pW212bE	5.69	97.3	0.228	17.7	
		7	42	40-48	pW104aE	pW108aH	4.18	59.9	-0.166	13.3	
		9	66	51-71	fito017b	pW187bH	4.59	81.4	-0.178	14.5	21
	GNT	5	83	79-85	fito100b	pX119dH	3.21	27.2	0.096	10.4	17.3
		7	2	0-12	fito472	pW225aD	3.32	19.9	0.054	10.8	
		8	44	42-51	fito204a	fito018	3.33	26.0	0.055	10.8	
	Aliphatic	2	89	82-95	fito375	fito034	4.62	75.1	-1.493	14.5	35.2
		3	100	95-100	BRMS015a	-	3.91	48.0	-1.593	12.4	
		7	43	41-48	BRMS042 & BRMS050	pW108aH	12.39	100.0	-2.603	34.3	
	Indolic	6	81	80-82	fito190	fito429 & pW217cE	3.61	42.1	-3.693	11.5	12.4
		8	48	45-53	fito018	pX130cD	4.62	48.2	1.200	14.5	

Additive effect was calculated as (P2-P1)/2; R^2% coefficient of determination of each QTL. Adj R^2%: determination coefficient of each trait.

Table S4.1: (Continued)

Plant organ	Trait	Linkage group	Peak Position	Confidence interval (cM)	Left mark	Right mark	LOD	Cross validation frequency (%)	Additive effect	R^2%	Adj R^2%
Flower buds	GIB	5	71	68-72	pW164aE	BRMS020	12.7	95.6	-0.456	34.9	33.8
	SIN	5	69	66-71	pW160dH	pW164aE	6.3	50.3	-0.333	19.2	21.2
		9	68	60-71	fito016 & fito017b	pW187bH	4.1	36.2	-0.266	12.9	
	GER	3	47	45-48	fito203c	pX111aD & fito040a	4.2	65.3	0.048	13.3	13.3
		9	63	50-70	fito016 & fito017b	pW187bH	6.0	98.6	0.040	18.4	
	GRA	9	61	55-65	fito016 & fito017b	pW187bH	9.6	99.2	1.938	27.8	19.5
	GNA	3	7	5-10	pW174aX & pW256aH	pW212bE	3.9	60.5	-1.106	12.3	41.2
		3	98	94-100	BRMS015a	pX146dH	5.0	47.0	-1.422	15.7	
		7	58	54-66	pW108aH	fito088b	5.9	68.8	-1.409	18.0	
		9	60	52-65	pW108gH	pW187bH	10.3	99.2	-1.717	29.5	
	PRO	3	7	5-9	pW174aX & pW256aH	pW212bE	14.5	95.8	1.664	38.8	34.2
		9	66	52-71	fito016 & fito017b	pW187bH	3.6	25.4	-0.622	11.5	
	OHGBS	2	66	63-69	pW120eX &fito161	Ol13-E08	4.9	73.2	0.094	15.3	12.7

Additive effect was calculated as (P2-P1)/2; R^2% coefficient of determination of each QTL. Adj R^2%: determination coefficient of each trait.

Table S4.1: (Continued)

Plant organ	Trait	Linkage group	Peak Position	Confidence interval (cM)	Left mark	Right mark	LOD	Cross validation frequency (%)	Additive effect	R^2%	Adj R^2%
	GBS	3	72	69-74	fito227	pW196aH, fito488, pX131bX, pX119bH & pW219aE	3.4	26.9	-0.668	10.8	41.1
		4	52	50-55	fito139b	fito132c	3.4	28.6	0.449	10.8	
		9	34	30-41	pW108gH	fito016 & fito017b	6.7	99.9	0.677	20.4	
	NeoGBS	6	81	80-82	fito190	fito429	5.5	80.1	-4.073	17.1	5.9
		8	50	45-53	fito018	fito146b	4.0	45.4	0.915	12.7	
	GNT	8	47	45-53	fito018	pX130cD	7.5	100.0	0.124	22.3	25.8
	Aliphatic	2	90	83-95	fito375	fito034	7.1	99.5	-1.679	21.3	46.6
		3	46	40-47	pW125dE	pW172aH	3.3	22.1	1.167	10.4	
		3	100	95-100	BRMS015a	-	5.3	91.1	-1.401	16.3	
		7	43	41-45	BRMS042 & BRMS050	pW108aH	20.3	100.0	-3.164	49.7	
	Indolic	6	81	80-82	fito190	fito429	3.8	78.0	-3.995	11.9	12.2
		8	48	45-53	fito018	pX130cD	3.3	22.7	1.131	10.7	
		9	12	10-13	pW189aX	pX140dX	3.3	37.2	2.848	10.6	
	Total	2	90	81-95	fito375	fito034	3.8	43.0	-1.853	12.0	14.8
		7	63	55-67	fito088b	pX110aE	4.4	67.0	-2.312	13.9	
Seeds	GIV	5	66	65-67	fito316, pX147fH, pX126bX, fito259, pW127cE & fito279a	pW160dH	8.01	64.2	23.121	24.2	1.7
		5	84	83-85	fito100b	fito279b & pW213bX	19.43	80.0	-26.917	49.0	

Additive effect was calculated as (P2-P1)/2; R^2% coefficient of determination of each QTL. Adj R^2%: determination coefficient of each trait.

Table S4.1: (Continued)

Plant organ	Trait	Linkage group	Peak Position	Confidence interval (cM)	Left mark	Right mark	LOD	Cross validation frequency (%)	Additive effect	R^2%	Adj R^2%
	GIB	5	73	71-74	BRMS030	pW198bH	15.64	99.6	-6.353	41.8	
		9	66	46-71	fito016 & fito017b	pW187bH	4.15	59.5	3.021	13.6	
	SIN	5	68	66-71	pW160dH	pW164aE	14.86	100.0	-7.372	40.2	45.1
		9	21	19-22	pW203dX, pW233aE & fito287a	pX147iH & pX146cH	4.44	67.7	-3.605	14.2	
		9	70	65-71	fito016 & fito017b	pW187bH	11.16	100.0	-5.846	32.1	
	GER	9	67	63-71	fito016 & fito017b	pW187bH	13.87	99.9	6.754	38.2	22.2
	GRA	4	84	78-92	fito017a	fito102	4.04	51.6	-6.794	13.1	47.5
		9	65	61-69	fito016 & fito017b	pW187bH	17.99	99.8	15.862	46.4	
	GNA	3	6	5-9	pX141bH	pW212bE	5.33	90.5	-16.807	16.9	43.9
		9	64	56-68	fito016 & fito017b	pW187bH	12.52	99.6	-24.759	35.2	
	PRO	3	8	7-10	pW174aX & pW256aH	pW212bE	18.45	98.5	33.357	47.2	42.7
		6	90	87-98	fito373b	fito040c	3.96	54.0	-11.643	12.8	
		9	63	46-71	fito016 &fito017b	pW187bH	5.32	94.0	-8.228	16.2	
	ALY	5	84	83-85	fito100b	fito279b & pW213bX	19.65	80.0	-42.963	49.4	0.6
	OHGBS	5	72	70-74	BRMS030	BRMS020	8.02	100.0	-1.899	24.3	43.1
		7	52	45-59	BRMS042 & BRMS050	pW108aH	7.87	99.9	1.964	23.8	
		8	52	45-53	pX130cD	fito373c	3.86	42.6	-1.231	12.5	

Additive effect was calculated as (P2-P1)/2; R^2% coefficient of determination of each QTL. Adj R^2%: determination coefficient of each trait.

Table S4.1: (Continued)

Plant organ	Trait	Linkage group	Peak Position	Confidence interval (cM)	Left mark	Right mark	LOD	Cross validation frequency (%)	Additive effect	R^2%	Adj R^2%
	GBS	1	94	87-95	fito426	pW248aX	4.40	71.5	0.563	14.1	36.7
		2	68	64-72	pW250bH, pWd251hX, pX128dX, pW148aE, fito237 & pW177cH	Ol13-E08	9.72	100.0	-0.809	28.6	
		4	44	42-48	pX111eD	pW193bE	4.00	58.9	1.569	12.9	
		5	62	56-65	pW209aH	fito156a, pX139dH, pX101aX & pW190cX	3.58	33.9	-0.765	11.7	
		5	85	84-86	fito294a	fito353	10.61	78.9	1.607	30.7	
		6	94	86-105	fito132b	fito040c	3.65	38.3	-0.520	11.9	
		7	76	74-78	pW192cE	pX126aX, BRMS040a & B.n.50F	6.69	99.7	0.851	20.7	
	NeoGBS	5	84	80-85	fito100b	fito279b & pW213bX	4.22	68.8	-0.490	13.6	10.4
		8	31	26-37	pWd251gBr	pW231aX	4.66	73.3	0.261	14.9	
	Aliphatic	7	43	40-48	BRMS042 & BRMS050	pW108aH	4.44	66.7	-2.026	14.0	28.0
		7	58	54-67	pW108aH	fito088b	7.93	95.8	-2.544	23.6	
	Indolic	8	51	50-59	pX130cD	fito146b	3.21	16.1	1.133	10.3	11.8
	Total	6	92	87-99	fito132b	fito040c	5.89	81.9	-31.215	18.4	16.9
		9	66	60-71	fito016 & fito017b	pW187bH	4.07	36.0	-14.543	13.1	

Additive effect was calculated as (P2-P1)/2; R^2% coefficient of determination of each QTL. Adj R^2%: determination coefficient of each trait.

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CAPÍTULO V

MODIFICATION OF LEAVES GLUCOSINOLATE CONTENT IN KALE (*BRASSICA OLERACEA* VAR. *ACEPHALA*) BY DIVERGENT SELECTION AND SIDE EFFECT ON FLOWER BUDS AND SEEDS

5. MODIFICATION OF LEAF GLUCOSINOLATE CONTENT IN KALE (*BRASSICA OLERACEA* VAR. *ACEPHALA)* BY DIVERGENT SELECTION AND SIDE EFFECT ON FLOWER BUDS AND SEEDS

Tamara Sotelo, Pablo Velasco, Pilar Soengas, Víctor M. Rodríguez, M. Elena Cartea

Group of Genetics, Breeding and Biochemistry of Brassicas, Department of Plant Genetics, Misión Biológica de Galicia, Spanish Council for Scientific Research (MBG-CSIC), Pontevedra, Spain.

5.1. ABSTRACT

Modification of the content of secondary metabolites opens the possibility of obtaining vegetables enriched in healthy compounds. Divergent mass selection is presented in this paper as a tool in plant breeding to generate groups of individuals that share the same genetic background but with extreme values for a particular trait. We report here the first results of a direct divergent selection for glucosinolate (GSL) content in order to develop six kale genotypes (*Brassica oleracea* var *acephala*) genotypes with divergent concentration of the three major GSL, sinigrin (SIN), glucoiberin (GIB), and glucobrassicin (GBS) in leaves. The aims of this study were to determine if the three divergent selections were successful in leaves and how each divergent selection affected the GSL content of the same GSLs in flower buds and seeds. In addition, the relationships between the modification of the content of these three major GSLs in other GSLs present in kales was studied to increase our knowledge on the GSL biosynthesis pathway. For the three divergent selections evaluated, significant differences among cycles were observed. We can conclude that the divergent mass selection for the SIN, GIB and GBS leaf content was successful. Furthermore, there was a side effect of divergent selection performed in leaves in the GSL content of flower buds and seeds. The modification of one specific GSL in leaves was related to a variation of other GSLs in the three organs. Indirect effects of divergent selection performed for the two aliphatic GSLs in the content of other GSLs suggest that different alleles of the locus GSL-ALK are responsible for the variation across the selection cycles.

Key words: Divergent mass selection, glucosinolates, *Brassica oleracea*, GSL-ALK.

5.2. INTRODUCTION

Glucosinolates (GSLs) are a major class of secondary metabolites found in the family *Brassicaceae.* Due to their enhanced plant protection to biotic and abiotic stresses (Fahey et al., 2001; Santolamazza Carbone et al., 2014) and their preventive effects on several human cancers (Fahey and Stephenson, 1999a; Forte et al., 2008), they have been extensively investigated. The hydrolytic breakdown products of GSLs, especially isothiocyanates (ITCs), have beneficial effects on human health, such as cytotoxic and apoptotic effects in damaged cells, preventing cancer in humans and reducing risk for degenerative diseases (D'Mello et al., 1993; Fahey et al., 1997; Rosa et al., 1997; Fahey and Stephenson, 1999a; Fahey and Talalay, 1999b; Cartea and Velasco, 2008; Forte et al., 2008; Van Horn et al., 2008; Virgili and Marino, 2008). In contrast, in rapeseed meal, the dominant GSL, progoitrin (2-hydroy-3-butenyl GSL, PRO) is changed into an oxazolidine-2-thione, which causes goiter and has other detrimental effects on animal health (Liu et al., 2012). Therefore, enrichment of beneficial GSLs and reduction of detrimental GSLs are given great attention in brassica crops breeding.

GSLs are sulfur-rich plant secondary metabolites with a basic skeleton consisting of a β-thioglucose residue, an N-hydroxy monosulfate moiety, and a variable side chain (Halkier and Du, 1997; Kliebenstein et al., 2001b). Generally, GSLs are divided into three different classes according to the amino acid precursor in biosynthesis and are called aromatic GSLs (derived from phenylalanine (Phe) or tyrosine (Tyr)), aliphatic GSLs (derived from methionine (Met), alanine (Ala), valine (Val), leucine (Leu) and isoleucine (Ileu)) and indolic GSLs (synthesized from tryptophan (Trp)) (Zukalova and Vasak, 2002; Bekaert et al., 2012).

The accumulation and profile of GSLs are highly dependent on the genotype, although it is also affected by environmental and developmental factors (Kliebenstein et al., 2001a; Brown et al., 2003). Therefore, knowledge on the genetics and heredity of GSLs in brassica crops is an important tool to design appropriate strategies to increase the content of those GSLs related to human health and plant protection. It is known that three loci mainly determine the profile and content of aliphatic GSLs in *B. oleracea*. The presence of 3C-GSL is controlled by a dominant allele of GSL-PRO whereas the presence of 4C-GSL and 5C-GSL is controlled by a dominant allele of GSL-ELONG. Another

major gene involved in the synthesis of aliphatic GSL is GSL-ALK, which controls the conversion of methylsulphinyl GSL into alkenyl GSL (Li et al., 2001), a step related with the production of sinigrin (2-propenyl, SIN) and gluconapin (3-butenyl, GNA). The indolic GSLs pathway is distinct from the aliphatic GSLs pathway and the biosynthetic indolic GSLs pathway is less studied than the aliphatic GSLs pathway. Nowadays, there are key loci that synthesized the core structure of indolic GSLs biosynthesis such as CYP79B2, CYP79B3 or CYP83B1 (Mikkelsen et al., 2000; Bak et al., 2001; Naur et al., 2003).

It is known that the concentration of GSLs shows a high variability among species, different varieties of the same species or even among plants of the same variety (Kushad et al., 1999). This genetic variability allows modifying the profile and increasing or reducing the GSLs concentration by selecting those plants with the desired phenotype or introducing desired characteristics by introgression. The increase of beneficial GSLs and the reduction of detrimental GSLs are a target in Brassica improvement in order to obtain crops with high value and improved food quality. The first modification of GSLs content by classical breeding took place in the 70s, when low erucic acid and low GSLs content varieties of *B. napus* were obtained by introgression from other *B. napus* cultivars (Stefansson and Kondra, 1975; Röbbelen and Thies, 1980). In the 90s, UK groups held a screening of diverse wild *Brassica* species and found that *Brassica villosa* contained a high concentration of glucoraphanin (4-methylsulphinylbutyl, GRA). This wild species was crossed with a commercial broccoli leading to the production of a new cultivar of broccoli enriched in GRA (Mithen et al., 2003; Sarikamis et al., 2006). There are other techniques to modify the concentration of GSLs. Liu et al., (2012) obtained *B. napus* seeds enriched in GRA thought the GSL-ALK silencing using RNAi.

Divergent mass selection has been widely used in plant breeding as it can generate groups of individuals that share the same genetic background but with extreme values for a particular trait. Stowe et al. (2011) used this type of selection to modify the total GSLs content of leaves of a rapid cycling variety of *B. rapa*. After three cycles, there were significant differences on GSLs concentration between the highest and lowest content genotypes with the control lines. This kind of selection could also be used to modify the content of a particular GSL. As it was previously explained, the content and profile of these secondary metabolites vary with plant species and plant organs (Brown at al., 2003;

Velasco et al., 2007). Selection carried out in one organ could produce side effects on the content of GSLs in other organs of the plant. Modification or selection by one gene of the GSLs biosynthetic pathway can also produce alterations or modifications in the concentration of other GSLs within the same biosynthetic pathway.

In kales (*Brassica oleracea* var. *acephala*), two aliphatic GSLs, SIN and glucoiberin (3-methylsulphinylpropyl, GIB), and one indolic GSL, glucobrassicin (3-indolylmethyl, GBS), are the predominant in the leaf profile (Cartea et al., 2008; Velasco et al., 2007). We report herein the results of three cycles of divergent mass selection for GIB, SIN and GBS content in leaves. This on-going selection program provides unique germplasm to study the direct and indirect effects of selection on individual GSLs concentration. Our objectives were: 1) studying the effect to the divergent selections for the content of two aliphatic GSLs (GIB and SIN) and one indolic GSL (GBS) in leaves, 2) determining the side effect of divergent selections in seeds and flower buds and 3) establishing whether the content of other GSLs may be altered with the selections carried on in leaves.

5.3. MATERIALS AND METHODS

5.3.1. Divergent selection program

Divergent selections were started in 2006 by using seeds of the kale population MBG-BRS0062, kept at the *Brassica* germplasm bank at Misión Biológica de Galicia (MBG-CSIC) (Galicia, NW Spain). The population presents variability for GSL concentration and this is a desirable characteristic to realize a mass divergent selection. These divergent selections were designed to obtain plant varieties with high (HSIN) and low (LSIN) sinigrin content, high (HGIB) or low (LGIB) glucoiberin content, and high (HGBS) or low (LGBS) glucobrassicin content. In 2006, approximately 750 plants from cycle 0 (C0) were transplanted in the field into six cages (125 plants each). The leaf GSL content of all the plants was assessed 120 days after sowing. In each cage, 20 plants with an extreme content of the relevant GSL (i.e. the highest or the lowest concentration) were selected (≈20% selection intensity). Cross-pollination among the selected plants in each cage was

obtained by using bumblebees (*Bombus terrestris*). In 2007, seeds were taken from the selected plants of the C0 and each divergent selection to create the cycle 1 (125 plants per cage). According to the protocol adopted for cycle 0, only those plants that showed extreme leaf GSL content were selected (20 plants per cage among 100 plants evaluated for GSL analyses). From 2008 to 2009, this process was repeated for two successive generation cycles. In 2010, approximately 150 plants for all cycles and for each divergent selection (C0, C1, C2 and C3 for each SIN, GIB and GBS) were grown in the greenhouse under controlled conditions. At 6-8 leaf stage, plants were transplanted to the field into isolate experimental plots to obtain the recombined genotypes in the same year (2011) and under the same environmental conditions.

5.3.2. Evaluation trials

Recombined plants from 19 cycles of divergent selection (three cycles for high and low SIN, GIB and GBS content) plus the original cycle (C0) were studied in the same year in order to avoid variations on GSLs content due to environmental conditions. The study was conducted during 2012 at MBG-CSIC (Galicia, NW Spain). Plants were grown in multi-pot trays under controlled conditions in an acclimatized greenhouse from July to August in 2012 (Figure 5.1). On 29th August plants were transplanted into the field (Salcedo, NW Spain, 42º 24'N, 8º 38'W) at 5-6 true leaf stage. Experimental design was a randomized complete block with three replicates. Each plot had two rows spaced 0.8 m and each row consisted of 15 plants spaced 0.6 m.

Figure 5.1: Plants grown into the greenhouse.

The evaluation of C0 with the same precision than the other cycles requires a considerably larger number of experimental plots, as this population contained 100% of the initial variability for GSL concentration. For this reason, three plots of the C0 were planted per block, while for the other genotypes one plot per block was planted. This variability was of less magnitude in the rest of cycles, because their starting variability had been reduced by the first cycle of selection. Cultivation operations, fertilization, and weed control were carried out according to local practices and crop requirements (Figure 5.2). Leaf samples were harvested in the same day, on ≈90 days old plants. The third leaf of a total of 20 healthy and competitive plants from each plot was chosen as plant material for GSLs analysis. Leaf samples were divided in two different bulks. To study whether the effect of the divergent selection performed in leaves affects the content of the three GSLs under study in flower buds, samples were collected from the same experimental plot as plant grew depending on the flowering time of each variety. In this case, 15 flower buds were collected and divided in three bulks from each plot. Tissue samples from leaves and flower buds were stored at −80 °C, freeze-dried and ground until GSLs analysis.

Figure 5.2: Experimental field during 2012

In the same way, to study whether the effect of the divergent selection performed in leaves affect the content of the three GSLs under study in seeds, five 100 mg bulks of the recombined seeds obtained in 2011 for each genotype, were ground and analyzed to study the GSLs profile and GSLs content.

5.3.3. GSL identification and quantification

Sample extraction and desulfation were performed according to Kliebenstein et al., (2001b) with minor modifications. Two microlitres of the desulfo-GSL extract for seeds and flower buds and three microlitres for leaves were used to identify and quantify the GSLs. The chromatographic analyses were carried out on an Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The UHPLC column was a C18 Atlantis® T3 waters column (3µm particle size, 2.1 x100 mm i.d.) protected with a C18 guard cartridge. The oven temperature was set at 30 ºC. Compounds were separated using the following method in aqueous acetonitrile, with a flow of 0.8 mL min^{-1}: 1.5 minutes at 100% H_2O, an 11 min gradient from 0% to 25% (v/v) acetonitrile, 1.5 min at 25% (v/v) acetonitrile, a minute gradient from 25% to 0% (v/v) acetonitrile, and a final 3 min at 100% H_2O. Data was recorded on a computer with the LabSolutions software (Shimadzu). All GSLs (the three major under selection and other minor GSLs present in leaf samples) were quantified at 229 nm by using SIN (sinigrin, monohydrate from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) and GBS (glucobrassicin, potassium salt monohydrate, from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) as external standard and expressed in µmol g^{-1} dry weight (DW). Calibration equations were made with, at least, five data points, from 0.34 to 1.7 nmol for SIN and from 0.28 to 1.4 nmol for GBS. The average regression equations for SIN, and GBS were $y = 148818x$ ($R^2 = 0.99$), $y = 263822$ x ($R^2 = 0.99$), respectively.

5.3.4. Statistical analysis

Combined analyses of variance across selection cycles for total and individual GSLs at each organ under study (leaves, flower buds and seeds) were computed using the PROC GLM of SAS program (SAS, 2011). Population means for the cycles of selection were compared using the Fisher protected Least Significant Difference test (LSD, $p \leq 0.05$). Besides, simple linear regression analyses were performed for the GSL implied in the three divergent selections (SIN, GIB and GBS) as dependent variables and cycles of selection as independent variables for each organ under study (leaves, flower buds and seeds). Moreover, simple linear regression analyses were performed in order to determine the response of the three divergent selections carried out in leaves with the same GSL in flower buds and seeds and the indirect response of the divergent selection carried out in leaves with other GSLs present in leaves, flower buds and seeds. The GSLs selected were the independent variables and the other GSLs as well as the sum of aliphatic, indolic and total GSLs were the dependent variables.

5.4. RESULTS AND DISCUSSION

5.4.1. Direct response to divergent selection for sinigrin, glucoiberin and glucobrassicin in leaves

Significant and positive simple linear regression coefficients across selection cycles for SIN (R^2=0.9684, $P \leq 0.0001$), GIB (R^2=0.9311, P=0.0004) and GBS (R^2= 0.6574, $P \leq 0.0001$) concentration were observed in leaves (Figure 5.3). Generally speaking, the response to divergent selection for the three GSLs was effective and linear in leaves; therefore, mass selection is an efficient way of increasing or decreasing the concentration of individual GSLs Stowe et al. (2011) obtained similar results in a divergent selection to modify the content of total GSLs in *B. rapa*. However, there are no studies to compare the results obtained in our work regarding a particular GSL.

After three cycles of divergent selection, a modification in the concentration of the aliphatic GSLs (SIN and GIB) and the indolic GSLs (GBS) under study was observed in

both senses of the divergent selection. The increase observed in leaves was 52.5% (P=0.0074) and 77.68% (P=0.0410) for SIN and GIB, respectively and the reductions observed were 51.9% (P=0.0322) and 45.33% (P=0.0385) for SIN and GIB, respectively. Meantime, the divergent selection performed for the leaf GBS content, was only successful and significant for decreasing the concentration, with a reduction of 39.04% (P=0.0248). The asymmetric response in a divergent selection program has been found before, for example in maize for leaf chlorophyll content, but the cause is still unknown (Korkovelos and Goulas, 2011). There are some possible causes to explain this effect such as differential selection, genetic asymmetry, selection for heterozygotes, inbreeding depression or maternal effects (Falconer, 1989).

The mass selection is an effective method for highly heritable traits. Although the estimates of heritability could not be calculated with the experimental design used in our work, according to the results obtained, we can conclude that heritability should be high enough. The heritability of a complex trait is controlled by the interaction of multiple genes and environmental factors. In this sense, Madsen et al. (2014) in *B. napus* and Márquez-Lema et al. (2009) in *B. carinata*, estimated the heritability for total GSLs in seeds with values of h^2=0.90 and h^2=0.58, respectively. In another study, Van Doorn et al. (1998) established the heritability for two aliphatic GSLs (SIN and PRO) in different cultivars of Brussels sprouts with values of h^2= 0.77 and h^2= 0.79, respectively.

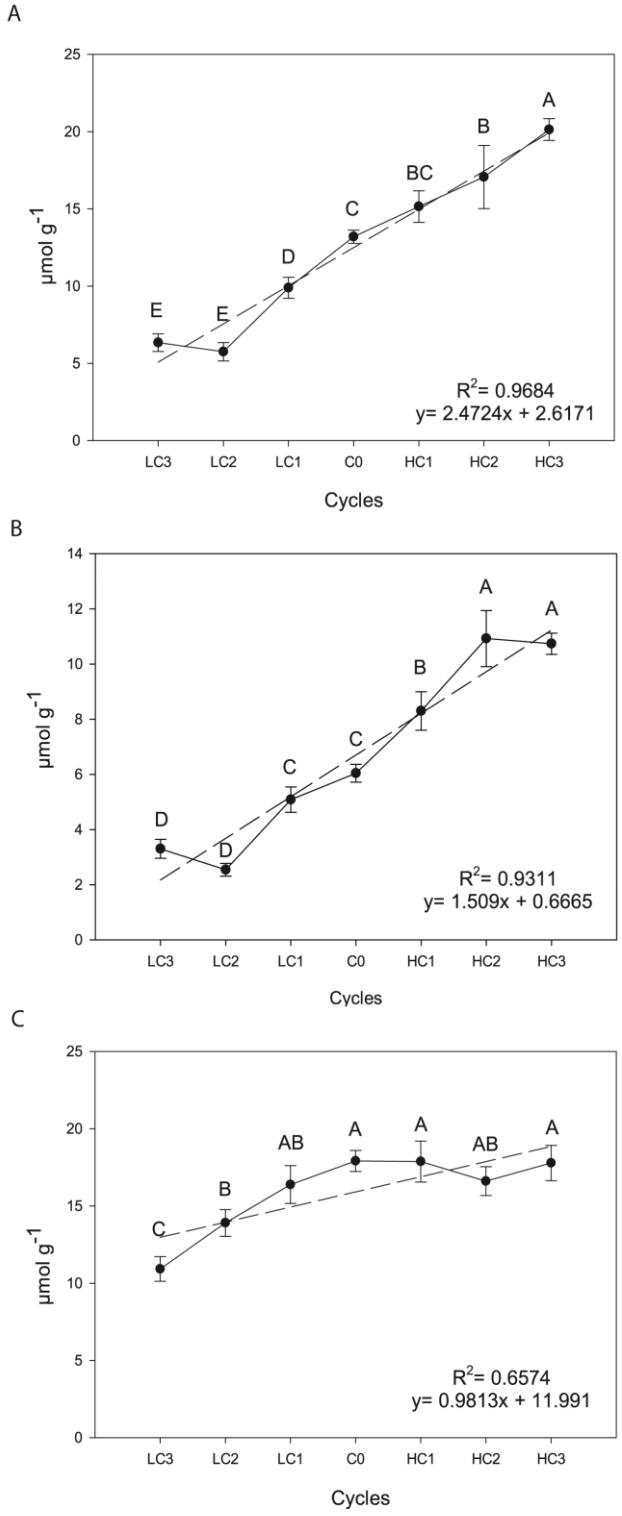

Figure 5.3: Graphical representations of simple linear regression divergent selection in leaves for the content (µmol g^{-1}) for sinigrin (A), glucoiberin (B) and glucobrassicin (C). ⊤: means standard error. LC1, low cycle 1; LC2, low cycle 2; LC3, low cycle 3; C0, original cycle; HC1, high cycle 1; HC2, high cycle 2; HC3, high cycle 3.

5.4.2. Response to divergent selection for sinigrin, glucoiberin and glucobrassicin in other organs

Leaves are the organ most consumed in kales, hence the importance to perform the divergent selections for specific GSLs in this organ. It has long been known that also there are GSLs in other organs such as roots, shoots, stems or seeds (Grubb and Abel, 2006) in part by the new GSLs biosynthesis or by translocation of the GSLs of leaves to other organs. We hypothesized that GSLs content on other organs, such as flower buds and seeds, could be affected by the selections performed in leaves; by these reason, GSL content was also determined in those organs.

There were significant and positive linear regression between the SIN concentrations modified in leaves and the concentration of SIN in flower buds and seeds. The same response was obtained in the other GSLs under selection, GIB and GBS although values of the R^2 for GBS were low (Table 5.1). Therefore, selection was effective not only in leaves, but also in flower buds and seeds.

There were significant differences among selection cycles for the three GSLs in flower buds (Figure 5.4). Significant and positive simple linear regression coefficients for SIN (R^2=0.8810, P=0.0017), GIB (R^2=0.8889, P=0.0015) and GBS (R^2=0.9838, P≤0.0001) across selection cycles were found (Figure 5.4). There was a 19.7% (P=0.0511) increase in SIN, a 79.62% (P=0.0461) increase in GIB and a 60.02% (P=0.0160) increase in GBS after three selection cycles versus the original cycle. Meantime, the decrease in the content for SIN was 42.73% (P=0.0153), 33.05% (P=0.0142) for GIB and 47.60% (P=0.0010) for GBS.

Positive and simple linear regressions were also found for SIN (R^2=0.6889 P=0.0208), GIB (R^2=0.6068, P=0.0390) and GBS (R^2=0.9677, P=0.0010) in seeds (Figure 5.5). For aliphatic GSLs, selection was successful to increase the SIN and GIB concentration but selection was unsuccessful for GBS. The increase was 123.23% (P=0.012) in SIN, and 661.78% (P≤0.001) in GIB and 53.35% (P=0.0584) in GBS relative to de C0, meantime the indolic GSLs was reduced in a 47.58% (P=0.0532) in GBS although there are no significant differences.

Table 5.1: Coefficients for simple linear regressions where sinigrin, glucoiberin and glucobrassicin in leaves are the independent variables and the other GSLs present in leaves, flower buds and seeds are the dependent variables.

		SIN			GIB			GBS		
		Leaves	**Flower buds**	**Seeds**	**Leaves**	**Flower buds**	**Seeds**	**Leaves**	**Flower buds**	**Seeds**
GIB	R^2	0.2072	0.0065	0.7698		0.7102	0.5055	0.0124	0.0405	0.0876
	a	-2.636	-0.2499	-0.838**		0.6513**	0.1758**	0.1230	0.1767	-0.0920
SIN	R^2		0.5511	0.3986	0.8022	0.5466	0.3050	0.2699	0.1627	0.0187
	a		0.7688**	0.1413**	-1.044**	-0.189**	-0.078**	1.0200	0.5091	-0.0176
GBS	R^2	0.0001	0.1770	0.0037	0.8621	0.1396	0.0808		0.2873	0.2687
	a	0.0167	0.7173*	-2.7113	0.639**	0.3307	-3.4549		0.2171**	7.527**
PRO	R^2	0.5642	0.0358	0.0223	0.1589	0.0191	0.6797	0.0871	0.0473	0.0580
	a	12.325*	1.5229	0.2861*	-5.552	-0.4538	-0.773**	3.3980	2.5197	0.0774
GRA	R^2	-	0.1082	0.3042	-	0.4500	0.4772	-	0.0452	0.0294
	a	-	-8.6995	-5.9581	-	6.6729**	5.0037**	-	2.0638	1.2440
GNA	R^2	0.0928	-	0.0614	0.4870	-	0.551	0.0040	-	0.0416
	a	-19.22	-	1.4190*	-28.623	-	-1.723**	3.6650	-	0.1844
OHGBS	R^2	0.4880	0.0151	0.1161	0.0167	0.0205	0.0433	0.7135	0.2969	0.0141
	a	32.822	-3.4938	0.9019	7.937	0.3094	-0.3005	0.4861**	-8.2618**	0.1726
NeoGBS	R^2	0.0923	0.0207	0.0021	0.5209	0.0040	0	0.9331	0.6181	0.1600
	a	-4.632	0.4645	-2.9566	4.004	0.1439	0.0295	3.202**	1.0279**	7.5880
GNT	R^2	0.2049	0.0030	0.0084	0.0035	0.0299	0.4134	0.1026	0.1974	0.0556
	a	-7.821	-1.5807	-2.1207	0.773	-2.5277	-8.888**	-4.7210	3.3897*	-2.5483
Aliphatics	R^2	0.9735	0.1245	0.0203	0.1863	0.0013	0.0060	0.1442	0.0191	0.0002
	a	1.101**	0.1571	0.02234	1.000	-0.0085	-0.0061	0.368	0.0292	0.0010
Indolics	R^2	0	0.0517	0.0168	0.8599	0.0041	0.0067	0.9961	0.1270	0.0291
	a	-0.008	0.1279	0.2802	0.581**	0.0166	-0.0797	0.762**	0.0853	0.1857
Total	R^2	0.7630	0.0875	0.0201	0.9105	0.0001	0.0065	0.7341	0.0614	0.0014
	a	0.8171*	0.0725	0.0208	0.545**	0.0011	-0.0058	0.438**	0.0279	0.0025

Aliphatic glucosinolates: GIB, Glucoiberin; SIN, Sinigrin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; Indolic glucosinolates: OHGBS, 4-hydroxyglucobrassicin; GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin: Aromatic glucosinolate: GNT, Gluconasturtiin. R^2: coefficient of determination of each glucosinolate. a: slope of the line.

* Significant at $P \leq 0.05$, and ** significant at $P \leq 0.01$.

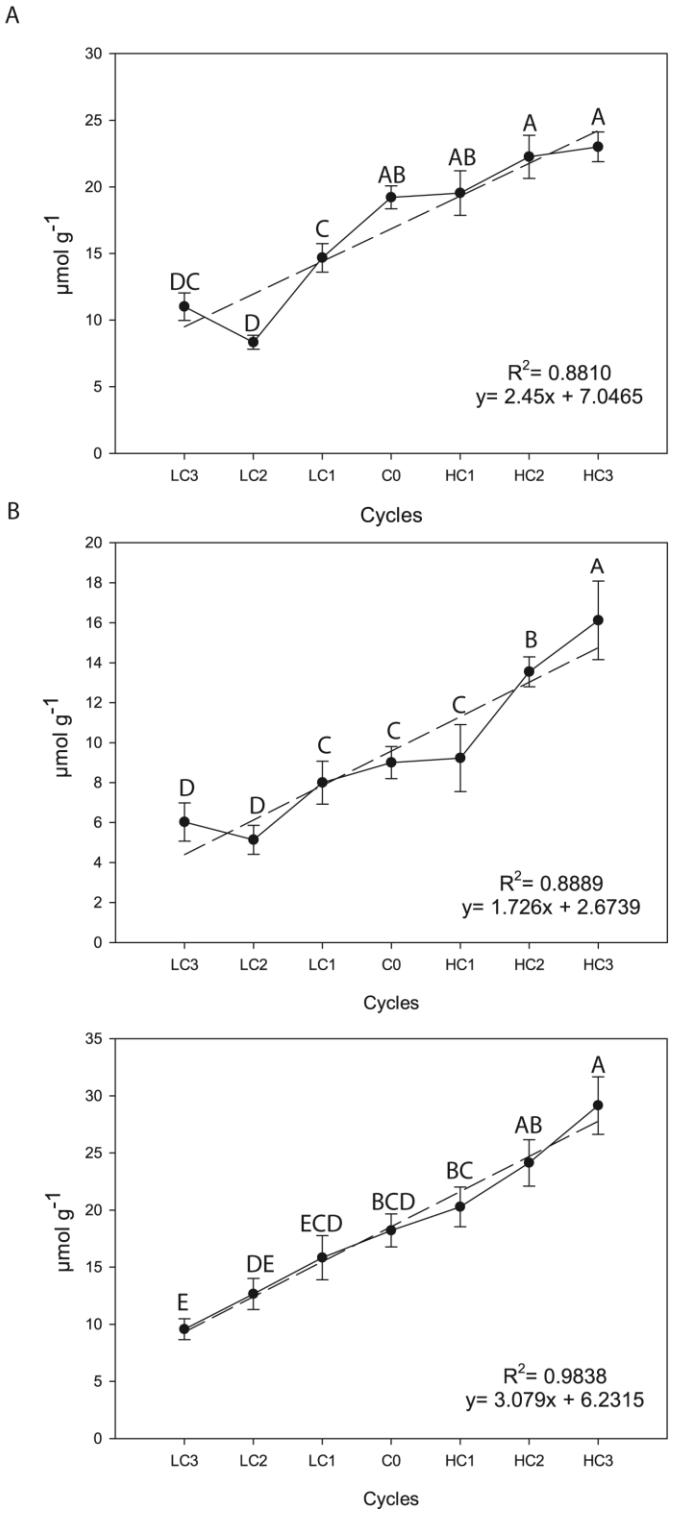

Figure 5.4: Graphical representations of simple linear regression divergent selection in flower buds for the content (µmol g^{-1}) for sinigrin (A), glucoiberin (B) and glucobrassicin (C). ┬: means standard error. LC1, low cycle 1; LC2, low cycle 2; LC3, low cycle 3; C0, original cycle; HC1, high cycle 1; HC2, high cycle 2; HC3, high cycle 3.

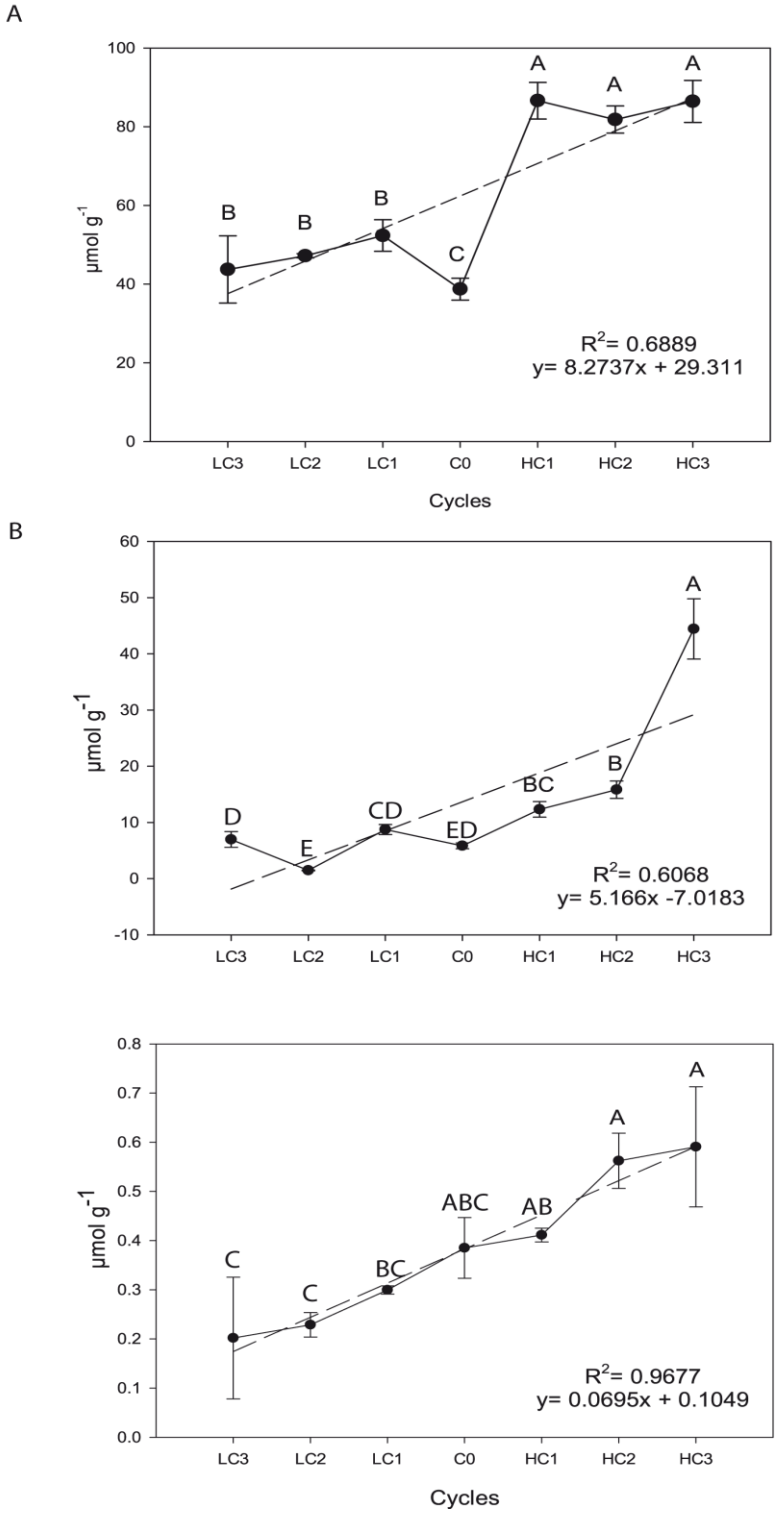

Figure 5.5: Graphical representations of simple linear regression divergent selection in seeds for the content (µmol g^{-1}) for sinigrin (A), glucoiberin (B) and glucobrassicin (C). ⊤: means standard error. LC1, low cycle 1; LC2, low cycle 2; LC3, low cycle 3; C0, original cycle; HC1, high cycle 1; HC2, high cycle 2; HC3, high cycle 3.

When selection is carried out to increase the content of the three GSLs in leaves, there is also an increase of the same GSLs in flower buds and seeds except for SIN in flower buds and GBS in seeds. When the selection is carried out for decreasing the content of the three GSLs in leaves, there is also a reduction of the same GSLs in flower buds and no related responses were found in seeds for both aliphatic and indolic GSLs. The reproductive organs, including seeds, flowers and fruits, which contribute most to plant fitness, are expected to have the highest concentrations of GSLs. In this way Brown et al. (2003) in *A. thaliana* demonstrate that seeds present higher content of GSLs than vegetative organs. GSLs accumulation represents the net effect of biosynthesis, transport and catabolism. Side effects of divergent selection performed in leaves could have an effect on the synthesis and transport of GSLs in flower buds and seeds. It can be possible that, by modifying the action of genes responsible for the concentration of GSLs in leaves, the action of the same genes were also modified in flower buds and seeds.

Differences in concentration and pattern of GSLs in different organs of *B. rapa* were related to differential expression of transcription factors involved in GSLs biosynthesis (Clarke, 2010). Since the same response was found in leaves, flower buds and seeds, genes related to biosynthetic pathway and no transcription factors could be implied in the divergent selection. Besides, there is a translocation of GSLs from vegetative organs to reproductive ones with the development. Du et al. (1998) observe that the high accumulation of GSLs in seeds is not connected with a corresponding high level of associated biosynthesis, suggesting the involvement of transport processes. Chen et al. (2001) demonstrated the translocation of radiolabeled p-hydroxybenzyl GSL from leaves to seeds via phloem, either exogenously applied or de novo synthesized. In fact, a recent study in *A. thaliana* shows the necessary presence of one specific transporter for the GSL translocation from other organs to seeds (Nour-Eldin et al., 2012) and the necessary presence of these transporters related with the movement of GSLs from roots to shoots (Madsen et al., 2014).

5.4.3. Indirect response to divergent selection on other GSLs

Besides the three major GSLs under selection, this population also presents other GSLs as the aliphatics progoitrin (PRO), glucoraphanin (GRA) and gluconapin (3- butenyl, GNA), the aromatic gluconasturtiin (2-phenethyl, GNT) and the indolics, hidroxyglucobrassicin (4-hydroxy-3-indolylmethyl, OHGBS) and neoglucobrassicin (1-methoxy-3-indolylmethyl, NEOGBS) which could have been modified indirectly by the divergent selection performed on leaves for SIN, GIB and GBS (Table 5.2).

A regression analysis was made with the leaf SIN, GBS and GIB content as independent variables and the content of the other GSLs in leaves, flower buds and seeds as dependent variables (Table 5.1). Significant and positive regressions were found between the leaf SIN content across selection cycles and PRO, aliphatic GSLs and total GSLs in leaves, GBS in flower buds and GNA in seeds. A negative correlation coefficient was found for GIB in seeds. In the biosynthetic pathway of GSLs, the locus GSL-ALK controls the side chain desaturation and its presence determines the production of the alkenyl GSLs SIN (3C-GSL), PRO and GNA (4C-GSL) (Li et al., 2001) (Figure 5.4A). By modifying the content of SIN, a positive related response was found in the content of PRO and GNA and a negative response in the content of GIB. These results suggest that modification in the SIN content by selection is related to the GSL-ALK locus. By modifying the content of SIN, a positive response is also found for GBS and total indolic GSLs. Recently, Sotelo et al. (2014) proposed that GSL-ALK plays a central role in the network for aliphatic GSLs in *B. oleracea* and stated that this gen also controls indirectly the variability for GBS content indicating a cross-talk between indolic and aliphatic pathways.

In the divergent selection program for leaf GIB content, significant and positive regressions were found between leaf GIB content and SIN and GBS, total indolic GSLs and total GSLs in leaves and GRA in flower buds and seeds (Table 5.1). Negative relationships were found between the leaf GIB content and PRO, GNA and GNT in seeds and SIN in both seeds and flower buds. In the biosynthetic pathway of aliphatic 3C-GSLs, the alkenization of GIB produces SIN. In the pathway of 4C-GSLs, the alkenization of GRA produces GNA, which is afterwards transformed into PRO. Alkenizations are carried out by the GSL-ALK locus.

Table 5.2: Glucosinolate (GSL) concentration (µmol g-1 dw) of the original cycle (C0) of the kale population for the three organs under study.

Glucosinolate	Leaves[2]	Flower buds	Seeds
GIB[1]	6.045	9.002	5.831
SIN[1]	13.202	19.219	38.724
GBS[1]	17.920	18.221	0.385
PRO	1.032	1.046	8.212
GRA	0.000	0.507	0.578
GNA	0.040	0.000	2.112
OHGBS	0.250	0.418	2.127
NeoGBS	2.698	4.178	0.292
GNT	1.717	1.261	0.305
Aliphatics	20.319	29.774	56.016
Indolics	20.868	22.816	2.804
TOTAL	42.905	53.852	59.125

[1] Glucosinolates studied in the three divergent selections. [2] Organ where selection was performed. Aliphatic glucosinolates: GIB, Glucoiberin; SIN, Sinigrin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; Indolic glucosinolates: OHGBS, 4-hydroxyglucobrassicin; GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin; Aromatic glucosinolate: GNT, Gluconasturtiin.

Our results showed that by altering the leaf content of GIB we found a negative response in the other organs in the content of SIN, PRO and GNA, and a positive response in the content of GRA, which suggests that the modification of the content of GIB in the divergent selection and the correlated responses are related to the major gene, GSL-ALK. Besides, the positive regression with the leaf GBS content also supports our assumption, as it was previously mentioned; GSL-ALK indirectly controls the variability for GBS, indicating a cross talk between indolic and aliphatic pathways. These results showed that we are probably selected by the GSL-ALK in both aliphatic divergent selections (Figure 5.4A). In the divergent selection for the leaf GBS content, significant

and positive regression were found with the content of the OHGBS, NEOGBS, total indolic GSLs and total GSLs. GBS is the precursor of OHGBS and NeoGBS in the biosynthetic pathway of indolic GSLs (Figure 5.4B); therefore, variation in GBS content provokes a positive response in the leaf content of NeoGBS and a negative response with OHGBS. In this case, we only found significant coefficients in leaves and flower buds, probably because the GBS levels in seeds are too low (Table 5.2).

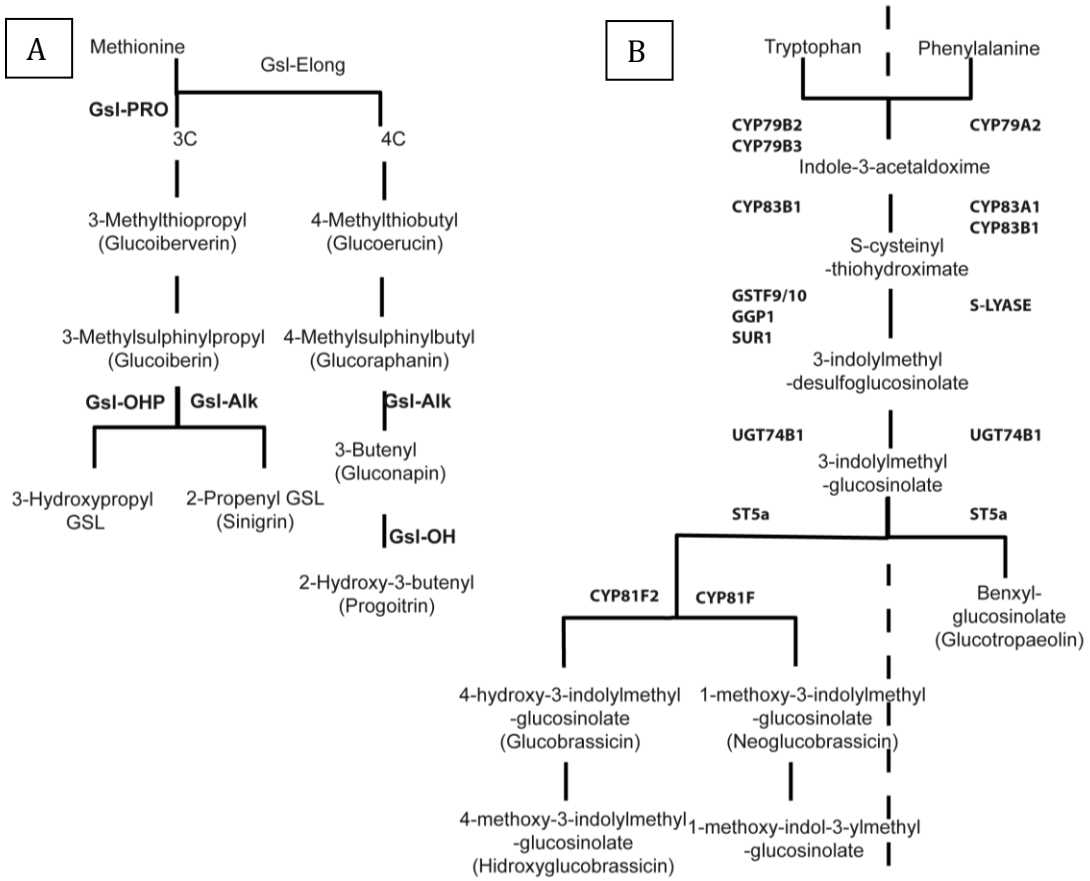

Figure 5.4: A biochemical genetic model of the biosynthesis of aliphatic glucosinolates (A) and indolic glucosinolates (B) in *Brassicaceae* including the major genes controlling this process.

Furthermore, a significant regression with the aromatic GSL GNT was found in flower buds although the R^2 was low. This relationship was not detected in the other organs, probably due to the higher concentration of GNT in flower buds than in leaves or seeds. Indolic and aromatic GSLs share several genes in their biosynthetic pathways

(CYP83B1, s-lyase, UGT74B1 or ST5a). It could be possible that when the content of GBS is modified by selection, we are acting in one of the cited genes (Fig. 4B), but further analysis would be necessary to identify which gene/s could be modified in the selection process. Unlike the case in the aliphatic divergent selections where we found a relationship between the modification in the aliphatic concentration and the indolic GSLs, in this case we could not find any relationship.

5.5. CONCLUSIONS

Divergent mass selection for the SIN, GIB and GBS leaf content was successful indicating that there are high genetic variability within the population which allows us modified the concentration of GSLs through mass selection. There was a side effect of divergent selection performed in leaves in the GSL content of flower buds and seeds, indicating modification of the synthesis of GSLs in these organs or translocation of GSLs from leaves.

Indirect effects of divergent selection performed for the two aliphatic GLS under selection (SIN and GIB) in the content of other GSLs suggest that different alleles of the locus GSL-ALK are responsible for the variation across the selection cycles. At the same time, this locus could be responsible of the indirect response found for the indolic GSL, GBS. More analysis should be necessary to identify which gene could be modified in the GBS mass selection.

The genotypes obtained in this study (with increased and decreased GSL content) can represent valuable materials for undertaking basic studies about the biological effect of the concentration of the major GSLs in kales.

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CAPÍTULO VI

DISCUSIÓN GENERAL

6. DISCUSIÓN GENERAL

Búsqueda de QTLs relacionados con los compuestos biosaludables (compuestos con actividad antioxidante y glucosinolatos)

Al igual que otras hortalizas del género *Brassica,* los diversos cultivos de *Brassica oleracea* representan una excelente fuente de compuestos bioactivos beneficiosos para la salud humana. Dentro de la gran variedad de estos compuestos, se encuentran diversos nutrientes en elevadas concentraciones tales como vitaminas, minerales, proteínas (Liu, 2004), diversos metabolitos antioxidantes y otros compuestos azufrados, exclusivos de las crucíferas y, denominados glucosinolatos (GSLs). Los compuestos antioxidantes y los GSLs exhiben diferentes propiedades biológicas relacionadas con la salud humana como el posible efecto preventivo frente al desarrollo de enfermedades relacionadas con procesos oxidativos como el cáncer y enfermedades neurodegenerativas y cardiovasculares. Este hecho ha conferido a las brásicas la categoría de "alimentos funcionales" (Fahey and Kensler, 2007) tan perseguidos hoy en día por parte del consumidor. Estas propiedades hacen que tanto los metabolitos con actividad antioxidante como los GSLs, sean caracteres interesantes a la hora de incluirlos en programas de mejora con el fin de obtener cultivos enriquecidos en estos compuestos.

Con el propósito de conocer la genética de estos caracteres e identificar las zonas genómicas que determinan su acumulación y variabilidad, se utilizó en esta tesis una población constituida por individuos dobles haploides (BoITBDH), derivada del cruce de un parental de ciclo rápido correspondiente a un cultivo de col china ("TO1000DH3") y una línea de brócoli ("Early big"), con el fin de realizar un análisis de QTLs para caracteres relacionados con la actividad antioxidante y el contenido en GSLs. Este análisis se realiza como un primer paso a la hora de estudiar el control genético de un carácter cuantitativo y constituye, por tanto, la primera etapa para poder identificar los posibles genes candidatos responsables de esa variación.

Existen un amplio número de métodos para evaluar la actividad antioxidante de los extractos vegetales *in vitro*. En esta tesis, se han comparado tres métodos de medida de actividad antioxidante total (ABTS, DPPH y FRAP) encontrándose que las correlaciones entre ellos fueron positivas y altamente significativas, aunque los valores

fueron moderadamente bajos. Estudios similares muestran valores de correlaciones superiores a los encontrados en este trabajo (Kusznierewicz et al., 2008; Zhi-Xiang et al., 2011; Soengas et al., 2012), si bien es importante destacar que el material empleado en esta tesis se encuentra emparentado genéticamente, ya que las líneas dobles haploides evaluadas derivan de un único cruzamiento. Estas bajas correlaciones, sugieren que aunque algunos de los métodos de medida puedan ser comparables por su tipo de reacción, se deberían aplicar diversos métodos para determinar la actividad antioxidante total. Se encontraron correlaciones positivas y altamente significativas entre los métodos de medida y el contenido en compuestos fenólicos totales, carotenoides y antocianinas. Esto confirmaría lo propuesto por otros autores como Krinsky et al. (2001) y Podsedeck et al. (2007), que postulan que estos compuestos son, en gran parte, los responsables de la actividad antioxidante total de los extractos obtenidos a partir de *B. oleracea*.

Para los métodos de medida de la actividad antioxidante, se encontraron 13 QTLs significativos, seis QTLs relacionados con los compuestos fenólicos totales, un QTL relacionado con antocianinas y tres QTLs con carotenoides. Se encontraron además tres regiones genómicas en C3, C5 y C7, que controlaban tanto la actividad antioxidante como el contenido en compuestos fenólicos y carotenoides, con lo cual se confirma la relación existente entre estos caracteres.

Además de los compuestos antioxidantes, se llevó a cabo un estudio genético sobre el grupo más importante de metabolitos secundarios presentes en las brásicas, los GSLs, al ser compuestos exclusivos de esta familia de plantas. El estudio se llevó a cabo en tres órganos diferentes: hojas, semillas y brotes florales. Cada parental y línea DH evaluada presentó el mismo perfil de GSLs independientemente del órgano estudiado, si bien difirieron en su concentración, siendo ésta mayor en semillas, seguida de brotes florales y hojas. Esta diferencia de concentración entre órganos de la planta había sido descrita anteriormente por otros autores como Brown et al., (2003) en *Arabidopsis thaliana* y Velasco et al. (2007b) en *B. oleracea*.

Los GSLs alifáticos fueron predominantes en los brotes florales y en las semillas llegando a representar el 93,3% de los GSLs totales en las semillas. En hojas, en cambio, fueron los GSLs indólicos los predominantes representando un 55,2% del total. Tras el análisis de QTLs, se detectaron un total de 82 QTLs, 40 de ellos para GSLs alifáticos, 17 para GSLs indólicos y 4 para GSLs aromáticos. Algunos de estos QTLs fueron comunes

para más de un GSL, por lo que se llevó a cabo un meta-análisis para calcular la posición de los QTLs consenso, fruto del cual se obtuvieron un total de 18 QTLs.

Como resumen del análisis de QTLs realizado en los capítulos III y IV se puede concluir que hemos detectado un total de 101 QTLs para compuestos biosaludables a lo largo del genoma de *B. oleracea*. El análisis fenotípico de los caracteres estudiados en los capítulos III y IV (métodos de medida de actividad antioxidante, compuestos fenólicos, carotenoides, antocianinas y GSLs), muestra que sus distribuciones son, en la mayor parte de los casos, transgresivas. Este tipo de segregación había sido descrito previamente en otros estudios relacionados con GSLs individuales (Mahmood et al., 2003; Ramchiary et al., 2007) y podría deberse a las nuevas combinaciones en las líneas dobles haploides de los alelos aditivos o bien a interacciones epistáticas entre los distintos loci de la población, lo cual demuestra la complejidad de las interacciones genéticas que dan lugar a estos caracteres.

Al comparar los resultados de los análisis de QTLs de los compuestos con actividad antioxidante y de GSLs, se encontraron tres regiones genómicas de la población BoITBDH, los cuales controlan la variabilidad y el contenido de compuestos con actividad antioxidante y GSLs (Tabla 6.1) en los cromosomas 3, 5 y 7. En las tres regiones genómicas el alelo que incrementa el carácter es aportado por el mismo padre. La realización una selección o mejora atendiendo a los genes de esa región, podría llevar a un incremento o disminución conjunta de estos metabolitos beneficiosos para la salud humana.

Dentro de las tres regiones encontradas, la región del grupo de ligamiento 7 se muestra como la de mayor interés debido a que controla variación para el contenido en GSLs totales y en fenólicos totales. En este caso, el incremento en los caracteres es dependiente de los alelos aportados por el parental To1000DH3 (Tabla 6.1). Las regiones de los grupos de ligamiento 3 y 5 controlan el contenido en algún GSL en particular y la actividad antioxidante medida con los métodos FRAP y DPPH respectivamente. Sería necesario realizar un mapeo fino de estas regiones para determinar si solo hay un locus o son varios loci ligados los que controlan la variabilidad para los caracteres indicados.

Tabla 6.1: Regiones del genoma de *B. oleracea* con QTLs que controlan el contenido de compuestos con actividad antioxidante y de compuestos GSLs

Grupo de ligamiento	Intervalo de confianza (cM)	Marcador de la izquierda	Marcador de la derecha	Parental que aporta el carácter	Caracteres implicados
3	5-12	pW174aX	Fito272	To1000DH3	FRAP y GNA
5	79-88	fito100b	fito353	Early Big	GBS, GNT y DPPH
7	57-76	pW108aH	pW192cE	To1000DH3	Fenólicos totales, GNA, GSLs alifáticos y GSLs totales

Diversos trabajos han puesto de manifiesto que la concentración y el perfil de los metabolitos secundarios varían en función de factores genéticos y ambientales. Entre los últimos, se incluyen diversos factores edafo-climáticos y de procesado además del órgano de la planta estudiado (Brown et al., 2003; Velasco et al., 2007; Soengas et al., 2012; Francisco et al., 2011). En esta tesis se han analizado diferentes órganos para evaluar dicha variación y estudiar la base genética del contenido en GSLs, de la actividad antioxidante y del contenido en metabolitos con actividad antioxidante en los distintos órganos de la planta. De acuerdo con nuestros resultados, se puede afirmar que las hojas poseen una mayor actividad antioxidante y un mayor contenido en metabolitos con actividad antioxidante que los brotes florales. Esto estaría en concordancia con estudios previos en *B. oleracea* acerca de la actividad antioxidante de los distintos órganos (Llorach et al., 2003; Soengas et al., 2012). Además, nuestros resultados también están de acuerdo con el estudio llevado a cabo por Francisco et al. (2009) en *B. rapa,* en el que se describe que las nabizas (hojas) presentan una mayor cantidad de compuestos fenólicos que los grelos (brotes). Al contrario de lo que ocurría con los compuestos con actividad antioxidante, el contenido total en GSLs fue mayor en brotes que en hojas, resultado que estaría de acuerdo también con estudios previos (Brown et al., 2003; Velasco et al., 2007b; Francisco et al., 2009) donde destacan que el contenido en GSL es mayor en los órganos reproductivos que en los vegetativos. Por todo esto, desde el punto de vista de la nutrición y los compuesto biosaludables, se podría recomendar el consumo de distintos

órganos de *B. oleracea*. El consumo de las hojas es recomendable por su elevado carácter antioxidante y el consumo de brotes florales por su elevado contenido en GSLs. La variación en la concentración de compuestos biosaludables según el órgano estudiado ha sido atribuido a distintos factores, como la síntesis diferencial según el tejido y la posterior traslocación, o el transporte de metabolitos secundarios dentro de la planta como ya ha sido demostrado en el caso de los GSLs por Chen et al. (2001) y Nour-Eldin et al. (2012).

En esta tesis se han encontrado QTLs específicos para cada órgano estudiado. En el capítulo III (relacionado con la actividad antioxidante), se encontraron cinco QTLs específicos para brotes y cinco para hojas, mientras que en el capítulo IV (relacionado con la síntesis de GSLs), de los 18 QTLs consenso, se encontraron dos QTLs específicos de semillas, dos QTLs específicos de brotes y un QTL específico de hojas. La detección de varios QTLs específicos en cada órgano en ambos trabajos abre la posibilidad de obtener variedades enriquecidas en un determinado compuesto y en un órgano concreto de la planta. Kliebenstein et al. (2001) encontró en *Arabidopsis* QTLs específicos para GSLs en distintos órganos (hojas y semillas). La existencia de QTLs específicos de cada órgano sugiere que la existencia de estos QTLs específicos vendría dada por el efecto cuantitativo del gen responsable de ese QTL y no por su efecto cualitativo (Kliebenstein et al., 2001).

Gracias al parentesco genético existente entre la planta modelo por excelencia *Arabidopsis thaliana* y nuestra especie bajo estudio, *B. oleracea,* se pudieron localizar mediante mapeo '*in silico*' genes relacionados con la ruta de síntesis de compuestos fenólicos y de GSLs en varios de los QTLs encontrados en esta tesis. En concreto, se han identificado algunos de los genes clave en la ruta de síntesis de los fenilpropanoides (ruta según la cual se sintetizan los compuestos fenólicos y las antocianinas) como por ejemplo, el gen HCT y el C3'H, identificados en el intervalo delimitado por los marcadores fito156c y pW125dE del cromosoma 3, región en la que se detectaron también QTLs para los tres métodos de medida de actividad antioxidante (FRAP, ABTS y DPPH). Por otro lado, se han logrado localizar los loci de mayor relevancia que controlan la mayor parte de la variabilidad en la ruta de biosíntesis de los GSLs alifáticos en *A. thaliana*, GLS-OH, GSL-PRO y GSL-ALK, en el intervalo de confianza de los QTLs consenso 3.1, 5.1 y 9.2, respectivamente. En el caso de los GSLs indólicos y

aromáticos, se han localizado los genes CYP79B2, CYP81F2, CYP79B3 y el ATR1 en el intervalo de confianza de los QTLs 1.2, 2.1, 7.4 y 8.1, respectivamente.

La variabilidad para un carácter puede estar determinada en parte por las relaciones epistáticas entre distintos QTLs, como es el caso de los GSLs analizados en este trabajo, siendo estas más numerosas y complejas para los GSLs alifáticos. Al contrario que en otros estudios previos llevados a cabo en *B. napus* (Feng et al., 2012), algunas de las interacciones encontradas son comunes a los tres órganos estudiados y, en este caso, son más numerosas en los brotes florales. Entre las relaciones epistáticas más importantes se encuentra la que existe entre los tres loci principales de la ruta de los GSLs alifáticos, GSL-ALK, GSL-PRO y GSL-OH. Estos resultados coinciden con el estudio realizado por Kliebenstein et al., (2009) en *A. thaliana*, en el que describe que las interacciones epistáticas entre estos tres loci determinan parte de la variabilidad de la concentración para los GSLs alifáticos. Además, en el trabajo realizado en esta tesis, se ha comprobado que el locus GSL-ALK ocupa el centro de una compleja red de interacciones epistáticas en donde participan hasta 12 QTLs relacionados con el contenido en GSLs alifáticos e indólicos. De acuerdo con nuestros resultados se podría sugerir que las rutas de síntesis de GSLs indólicos y alifáticos no son del todo independientes.

Todos los genes candidatos propuestos en este estudio podrán ser estudiados en profundidad y corroborados en breve, debido a que uno de los parentales que forman la población de mapeo empleada, TO1000DH3, ha sido utilizado en el estudio de secuenciación de *B. oleracea* cuya secuencia, todavía incompleta, (http://plants.ensembl.org/Brassica_oleracea/Info/Index) acaba de ser publicada recientemente. Esta publicación, permitirá en un futuro cercano corroborar los resultados encontrados en el estudio de sintenia, así como la búsqueda mucho más rápida y directa de otros genes implicados en la ruta de biosíntesis de fenilpropanoides y GSLs.

Modificación del contenido de glucosinolatos

Debido a las propiedades destacadas de determinados GSLs relacionadas con la defensa de la planta y con la salud humana, existe un creciente interés por desarrollar materiales

con contenidos elevados en un determinado GSL. La puesta en práctica de métodos para modificar el perfil de GSLs en los cultivos nos proporcionaría diferentes ventajas como incrementar aquellos GSLs que aportan beneficios específicos sobre la salud (Fahey et al., 1997; Fahey y Stephenson, 1999; Fahey y Talalay, 1999; Cartea and Velasco, 2008; Forte et al., 2008; Van Horn et al., 2008; Virgili and Marino, 2008) y reducir aquellos GSLs considerados perjudiciales tanto para la salud humana como para la animal, como es el caso del producto de degradación de la PRO, que puede llegar a causar bocio en animales (Liu et al., 2012).

Tras el análisis de GSLs en las tres selecciones divergentes diseñadas a partir de una población local de berzas (*Brassica oleracea* var. *acephala*), se pudo concluir que la selección llevada a cabo en hojas fue eficaz tanto para incrementar como para disminuir el contenido en GSLs, confirmando que la concentración de estos compuestos es un carácter con una elevada heredabilidad. El método de selección masal llevado a cabo en este trabajo, aunque lento, es un método de mejora clásica relativamente sencillo de realizar e ideal para caracteres con una elevada heredabilidad, pero su mayor ventaja es que no supone un rechazo por parte del consumidor como en el caso de la obtención de cultivos mejorados mediante transgénesis. Por otro lado, la obtención de un material con alto y bajo contenido en un GSL específico ofrece la oportunidad de disponer de un material útil en el estudio exhaustivo de las propiedades biológicas de estos compuestos, como puede ser el estudio de las propiedades antibióticas de los GSLs sobre distintas plagas y enfermedades de brásicas, el efecto en el rendimiento y producción de biomasa o el estudio *in vitro* de sus efectos biológicos sobre determinadas líneas de cáncer.

Existe un trabajo previo en el cual se utilizó un programa de selección divergente para modificar el contenido total de GSLs en hojas de una variedad de ciclo rápido de *B. rapa* (Stowe y Marquis, 2011). No obstante, el trabajo llevado a cabo en esta tesis constituye el primer estudio donde se comprueba la eficacia de la selección masal en la modificación del contenido en un GSL en particular. Cabe destacar que tras tres ciclos de selección, no se detectan signos de que la variabilidad genética se haya agotado en ninguna de las direcciones, por lo que cabría la posibilidad de desarrollar con éxito nuevos ciclos de selección.

Las selecciones llevadas a cabo en las hojas modificaron también el contenido en GSLs en otros órganos de la planta como los brotes florales y las semillas. Se comprobó

además que el hecho de alterar la concentración de un GSL en particular tiene un efecto, en mayor o menor medida, sobre el resto de GSLs de la planta. Por lo tanto, es de especial importancia conocer los genes bajo selección, para poder diseñar una estrategia que permita modificar solo el contenido en el GSL o GSLs de interés. Este resultado cobra una importancia relevante en nuestro estudio, ya que la relación existente entre un GSL seleccionado con el resto de GSLs, nos permitiría obtener y diseñar variedades diferentes a partir de un mismo fondo genético.

El efecto indirecto observado en las selecciones divergentes de los dos GSLs alifáticos (SIN y GIB) sobre otros GSLs, sugiere que diferentes alelos del locus GSL-ALK podrían ser los responsables de la variación de concentración entre los tres ciclos de selección. Comparando los trabajos de GSLs llevados a cabo en los capítulos IV y V de esta tesis, se puede concluir que el locus GSL-ALK juega un papel clave directo en la síntesis de los GSLs alifáticos e indirecto en la síntesis de los GSLs indólicos a través de diversas relaciones epistáticas. Además, nuestros resultados sugieren que este locus tiene un papel regulador sobre otros QTLs que controlan la variabilidad para el contenido en GSLs. Por todo ello, resulta obvio que el locus GSL-ALK es un gen candidato a tener en cuenta en los programas de mejora destinados a modificar el contenido en GSLs. Al parecer, las funciones de este locus no solo están relacionadas con la síntesis de GSLs, sino que además la variación en el locus GSL-ALK está relacionada con otros efectos de gran interés como el ritmo circadiano o la floración dependiente de la enzima AOP2 (Kerwin et al., 2011).

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CAPÍTULO VII

CONCLUSIONES

7. CONCLUSIONES FINALES

De acuerdo con los resultados expuestos en los capítulos anteriores, la presente Tesis Doctoral ha dado lugar a las siguientes conclusiones parciales de acuerdo con cada uno de los objetivos propuestos:

1. Identificación de QTLs relacionados con la actividad antioxidante en *Brassica oleracea*.

    1.1. Cuando se evalúa un material genéticamente relacionado, es necesario aplicar más de un método de medida a la hora de determinar la actividad antioxidante de una variedad debido a la baja correlación existente entre los diferentes métodos.

    1.2. El análisis de QTLs confirma que la actividad antioxidante está relacionada con el contenido en compuestos fenólicos así como con el contenido en carotenoides en los extractos metanólicos de Brásicas.

    1.3. Se proponen algunos genes candidatos relacionados con la ruta de síntesis de los fenilpropanoides basándonos en el estudio fenotípico de la actividad antioxidante de las líneas dobles haploides evaluadas y de su posterior sintenia con *Arabidopsis thaliana*.

2. Identificación de QTLs metabólicos y genes candidatos para la síntesis de glucosinolatos en *Brassica oleracea.*

    2.1. Los QTLs identificados para la variación de glucosinolatos en tres órganos (hojas, brotes florales y semillas) muestran la existencia de varios locus relacionados con la síntesis de glucosinolatos.

    2.2. Se proponen posibles genes candidatos de la ruta principal de biosíntesis para diferentes QTLs basándonos en el estudio fenotípico de las líneas dobles haploides de *Brassica oleracea* y de su sintenia con *Arabidopsis thaliana*.

2.3. Se detectaron un elevado número de interacciones epistáticas entre los distintos QTLs, siendo algunas de ellas comunes en los tres órganos. Se destaca el papel del locus GSL-ALK en la determinación de la variación de glucosinolatos alifáticos y se sugiere un efecto regulador de este locus.

3. Modificación del contenido de glucosinolatos en hojas de berza (*Brassica oleracea* var. *acephala*) mediante selección divergente y su efecto en brotes florales y semillas.

3.1. Las selecciones divergentes llevadas a cabo para los tres glucosinolatos mayoritarios, sinigrina, glucoiberina y glucobrasicina, en las hojas tras tres ciclos de selección son eficaces, indicando que el contenido en glucosinolatos es un carácter altamente heredable y que puede ser modificado mediante métodos de selección convencional. Las variedades obtenidas se presentan como un material idóneo para llevar a cabo estudios sobre los efectos biológicos de la concentración de estos glucosinolatos en los cultivos de brásicas.

3.2. Se encontró un efecto secundario de la selección divergente realizada en las hojas en el contenido de glucosinolatos en brotes florales y semillas, indicando una modificación en la síntesis de glucosinolatos en estos órganos o una traslocación de los GSLs sintetizados en las hojas a otros órganos de la planta.

3.3. Los efectos indirectos de la selección divergente realizada en hojas para los dos glucosinolatos alifáticos (sinigrina y glucoiberina) sobre el contenido de otros glucosinolatos de la planta, sugieren que diferentes alelos del locus GSL-ALK son los responsables de la variación a través de los ciclos de selección. Este locus podría ser además el responsable de la respuesta indirecta observada para el glucosinolato indólico. Serían necesarios más análisis para identificar el gen que se está seleccionando por contenido en glucobrasicina.

CAPÍTULO VIII

ANEXO

Publicaciones y descripción de las revistas científicas

Los resultados y conclusiones obtenidos en este trabajo de Tesis Doctoral han dado lugar a tres artículos científicos, dos de ellos se encuentran publicados en revistas científicas de carácter internacional incluidas en el SCI (Science Citation Index) y el tercero se encuentra en redacción. A continuación se detallan dichos artículos y el factor de impacto de las revistas científicas:

Artículos publicados:

Sotelo T, Soengas P, Velasco P, Rodríguez VM, Cartea ME. 2014. Identification of metabolic QTLs and candidate genes for glucosinolate synthesis in *Brassica oleracea* leaves, seeds and flower buds. PlosOne 9(3):e91428.

Sotelo T, Cartea ME, Velasco P, Soengas P. 2014. Identification of antioxidant capacity-related QTLs in *Brassica oleracea*. PlosOne 9(9): e107290.

Artículo en redacción:

Sotelo T, Soengas P, Velasco P, Rodríguez VM, Cartea ME. Modification of leaf glucosinolate content in kale (*Brassica oleracea* var. *acephala*) by divergent selection and side effect on flower buds and seeds.

Factor de impacto de las revistas:

PLOS ONE es una de las revistas científicas más importante dentro de su grupo. El factor de impacto de esta revista en 2013 fue de 3,5 encontrándose en la posición número 8 dentro de la categoría Multidisciplinar Science de un total de 56 revistas, por lo que es una revista del primer cuartil.

CAPÍTULO IX

PUBLICACIONES ORIGINALES

OPEN ACCESS Freely available online

PLOS ONE

Identification of Antioxidant Capacity -Related QTLs in *Brassica oleracea*

Tamara Sotelo*, María Elena Cartea, Pablo Velasco, Pilar Soengas

Group of Genetics, Breeding and Biochemistry of Brassicas, Department of Plant Genetics, Misión Biológica de Galicia, Spanish Council for Scientific Research (MBG-CSIC), Pontevedra, Spain

Abstract

Brassica vegetables possess high levels of antioxidant metabolites associated with beneficial health effects including vitamins, carotenoids, anthocyanins, soluble sugars and phenolics. Until now, no reports have been documented on the genetic basis of the antioxidant activity (AA) in *Brassica*s and the content of metabolites with AA like phenolics, anthocyanins and carotenoids. For this reason, this study aimed to: (1) study the relationship among different electron transfer (ET) methods for measuring AA, (2) study the relationship between these methods and phenolic, carotenoid and anthocyanin content, and (3) find QTLs of AA measured with ET assays and for phenolic, carotenoid and anthocyanin contents in leaves and flower buds in a DH population of *B. oleracea* as an early step in order to identify genes related to these traits. Low correlation coefficients among different methods for measuring AA suggest that it is necessary to employ more than one method at the same time. A total of 19 QTLs were detected for all traits. For AA methods, seven QTLs were found in leaves and six QTLs were found in flower buds. Meanwhile, for the content of metabolites with AA, two QTLs were found in leaves and four QTLs were found in flower buds. AA of the mapping population is related to phenolic compounds but also to carotenoid content. Three genomic regions determined variation for more than one ET method measuring AA. After the syntenic analysis with *A. thaliana*, several candidate genes related to phenylpropanoid biosynthesis are proposed for the QTLs found.

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* Email: tsotelo@mbg.csic.es

Introduction

Brassicaceae plants represent one of the major vegetable crops grown worldwide, with *Brassica oleracea* L. (2n = 18) as the main *Brassica* species consumed in Europe and the USA. Cruciferous vegetables, in particular those included in the *Brassica* genus, are an important part of the diet as they provide a multitude of nutrients and bioactive compounds [1]. A high consumption of *Brassica* vegetables reduces the risk of age-related chronic illnesses, degenerative diseases [2] and several types of cancer [3]. Human health benefits associated to *Brassica* consumption could be attributed, in part, to the large amount of constituents having strong antioxidant activity (AA). In fact, AA of *Brassica* vegetable extracts is higher compared to that of other vegetable crops like green pepper, carrot, potato or green bean [4]. Antioxidants have long been recognized to have protective functions against oxidative damage and are associated with a reduced risk of chronic diseases [5]. *Brassica* vegetables possess high levels of antioxidant metabolites associated with beneficial health effects, including vitamins (especially vitamin A, C, E, K and B-6), carotenoids (such as γ- and β-carotene and zeaxanthin), anthocyanins, folate, soluble sugars and phenolic compounds which are known to be the major antioxidants of *Brassica* crops [6–14].

Due to the complexity of food composition, separating each antioxidant compound and studying it individually is costly and inefficient. In addition, there might be synergistic interactions among the antioxidant compounds [15]. There are numerous methods for measuring the total AA of a plant extract *in vitro*. The 2- single electron transfer reaction based assays (ET) measure the reducing capacity of the samples. The ET group includes different methods like the ferric ion reducing antioxidant power assay (FRAP), and the AA measured with the reagents ABTS (2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and DPPH (2, 2-diphenyl-1-picrylhydrazyl), among others [15]. Generally speaking, correlations found among these three methods are high in Brassica extracts. Soengas *et al.* [16] found that the correlation between DPPH and FRAP was 0.8 when analyzing several *B. oleracea* crops. Kusznierewicz *et al.* [17] found a correlation of 0.96 between ABTS and DPPH in white cabbage. Zhi *et al.* (2011) [18] found correlations ranging from 0.76 to 0.82 among the three cited methods when analyzing different vegetables, including broccoli. In most studies, several ET methods are often used in order to measure the AA of a sample, but theoretically it could be possible to choose only one because of the high correlations among assays.

Phenolic compounds are known to be the major group with antioxidant capacity in *Brassica* crops [13]. These compounds are able to scavenge reactive oxygen species due to their electron donating properties. The most widespread and diverse group of polyphenols in *Brassica* species are flavonoids and hydroxycin-

namic acids. In many *in vitro* studies, phenolic compounds demonstrated higher AA than other antioxidants, such as vitamins and carotenoids [19].

Several studies have demonstrated that highly pigmented cultivars of some vegetables (i.e. cabbage, cauliflower) possess stronger AA than their respective light-colored cultivars [20–22]. This could indicate that pigments '*per se*' have AA. Carotenoids are a diverse group of more than 600 natural pigments that accumulate in the plastids of some vegetables leaves, flowers and fruits [23]. Some carotenoids are essential nutrients for humans, while others have protective effects against several diseases. Anthocyanins are natural pigments responsible for the blue, purple, red and orange colors in the major parts of all higher plants and have attracted much interest due to their impact on the sensorial characteristics of food products, as well as their health-related properties through various biological activities [24,25]. The AA of *Brassica* crops has been mainly related to phenolic compounds and vitamin C. However, carotenoids and anthocyanins could also play an important role.

Comparisons of *in vitro* AA of the main *B. oleracea* crops demonstrated that broccoli, kale and red cabbage show high AA [17,26]. Soengas *et al.* [16] compared the AA of six *Brassica* crops, including broccoli, cabbage, cauliflower, kale, nabicol and tronchuda cabbage, at four different plant stages with DPPH and FRAP assays. They found that kale and broccoli had the highest AA. Nilson *et al.* [27] found that AA of curly kale was at least 10-fold higher than that of cauliflower and white cabbage. At present, there are many studies about AA of *Brassica* crops because of the health related properties of antioxidants. However, as far as we know, there are no repots about genetics and heredity associated with AA in the *Brassica* genus.

QTL analysis is a very important tool in order to study the genetic base of AA. For the last decades, quantitative trait mapping has been the most common approach in order to analyze complex traits and measure the association of genetic markers with phenotypic variation. Identification of QTLs is essential for the understanding of the quantitative genetic control of AA and it is an early step in order to identify and estimate the gene number controlling each trait variation. The high co-linearity between *A. thaliana* and *Brassica* species can be used for identifying candidate genes underlying QTLs that affect AA. To our knowledge, this is the first report on the genetic basis of AA in *Brassica* crops. In other crops, only Jin *et al.* [28] in rice, Dobson *et al.* [29] in raspberry and Hayashi *et al.* [30] in lettuce studied QTLs for total water soluble AA and total phenolic, anthocyanin and carotenoid contents.

For this reason, the aims of our research were 1) to study the relationship among different ET methods for measuring AA, 2) to study the relationship between these methods and phenolic, carotenoid and anthocyanin contents and 3) to find QTLs of AA measured with ET assays and for phenolic, carotenoid and anthocyanin contents in two organs of a DH population of *B. oleracea* as an early step in order to identify genes related to these traits.

Materials and Methods

Chemicals

DPPH (2,20-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-striazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), hydrochloric acid, phenolics reagent, ABTS (2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)), potassium persulphate and gallic acid were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); ferric chloride and methanol were obtained from Panreacquimica S.A. (Castellar del Vallés, Spain).

Plant material and growing environments

The double haploid (DH) mapping population employed in this study (BolTBDH) was created from an F_1 individual, derived by crossing a DH broccoli line 'Early Big'(P_2) and a DH rapid cycling of Chinese kale line (TO1000DH3,P_1) [31]. Parents and 155 DH lines were grown in autumn 2011 (from September to November) and stored in the greenhouse under controlled conditions: 16 h of daylight and a temperature of 24±2°C; 8 h of darkness having 18±2°C at night; and a relative humidity of 55% in order to obtain enough seed in the same environmental conditions. Plants were sown in a completely randomized experiment with two replications and four plants per replication. Two sample types were collected and analysed: leaves (one month after sowing) and flower buds (taken sequentially depending on the maturity of each line). Bulks of individual samples were taken from each replication. Samples were frozen *in situ* in liquid N_2, immediately transferred to the laboratory and frozen at −80°C. All samples were freeze-dried (BETA 2–8 LD plus, Christ) for 72 h. The dried material was powdered by using an IKA-A10 (IKA-Werke GmbH & Co.KG) mill, and the fine powder was used for methanolic extractions.

Evaluation of AA

Freeze-dried and ground samples (10 mg) were extracted with 1 ml of 80% aqueous methanol in dark maceration for 24 h. After centrifugation (3700 rpm, 5 min), methanolic extracts were employed in order to determine AA (FRAP, DPPH and ABTS) of the mapping population. All AA assays and the content of metabolites with AA were carried out spectrophotometrically by using a microplate spectrophotometer (Spectra MR; Dynex Technologies, Chantilly, VA). Two repetitions were made for each sample and analysis. Standards prepared with different concentrations of Trolox (0, 0.008, 0.016, 0.024, 0.032, 0.04 mM) were measured for FRAP, DPPH and ABTS analyses and AA values were normalized to Trolox equivalents per gram of dry weight.

FRAP assay

The ferric reducing antioxidant activity (FRAP) assay of Benzie and Strain [32] was measured in all samples. Fresh FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6), one volume of 10 mM TPTZ in 40 mM hydrochloric acid and one volume of 20 mM ferric chloride, and then incubating at 37°C for 5 minutes. For each analysis, 30 µl of methanolic solution of the two organs (leaves and flower buds) were added to 20 µl of distilled water and 250 µl of fresh FRAP solution and mixed thoroughly. The increase in absorbance was recorded at 593 nm after 20 min.

DPPH radical scavenging activity

The antioxidant activity by the DPPH method was determined by monitoring the disappearance of the radical DPPH spectrophotometrically, according to Brand-Williams *et al.* [33]. The working DPPH reagent was prepared by dissolving DPPH in methanol to a final concentration of 75 µM. Fifty microliters of extract for leaves and 35 µl for flower buds were added to 250 µl of freshly prepared DPPH reagent and mixed thoroughly. Readings were taken at 517 nm after 30 min of incubation in the dark at room temperature.

ABTS+ radical scavenging activity

The method of decolorization of free radical ABTS+ employed was a modified version of that used by Samarth *et al.* [34] and initially reported by Re *et al.* [35]. ABTS+ was generated by oxidation of ABTS 7 mM with potassium persulphate 2.45 mM in water, at room temperature for 16 h. For each analysis, the ABTS+ solution was freshly diluted with water in order to obtain an initial absorbance around 0.8 at 734 nm. An aliquot of 20 µl methanolic extract for leaves and 30 µl for flower buds were added to 250 µl of ABTS+ solution. Absorbances were measured at 734 nm after 30 min of incubation in the dark at room temperature.

Quantification of phenolic content

The total phenolic content of the extracts was determined according to the phenolic colorimetric method described by Dewanto *et al.* [36]. The same methanolic extracts employed for AA assays were employed in order to determine phenolic content. Extracts were oxidized with 50 µl of 0.5 M Folin reagent. After 5 min, 200 µl of a 20% Na_2CO_3 solution were added in order to neutralize the reaction. Absorbances were measured at 760 nm after 2 h of incubation in the dark at room temperature. Standards prepared with different concentrations of gallic acid (0, 0.008, 0.016, 0.024, 0.032 and 0.04 mM) were also measured. Results were expressed in terms of micromoles of gallic acid equivalents per gram of dry weight.

Quantification of carotenoid content

Carotenoid content was determined according to Sims & Gamon [37] with minor modifications. Lyophilized samples (10 mg) were ground in 1 ml cold acetone/Tris buffer solution (80:20 vol:vol, pH = 7.8). Samples were mixed overnight in the dark at room temperature; afterwards, the absorbance of samples was measured at 537, 647 and 663 nm. Carotenoid content was computed by following the equations of Sims & Gamon [37] and results were expressed in micromoles per gram of dried weight.

Quantification of anthocyanin content

Anthocyanin content was determined according to Murray *et al.* [38] with minor modifications. Lyophilized samples (10 mg) were ground in 1 ml of cold methanol/HCL/water (90:1:1, vol:vol:vol). Samples were mixed overnight in the dark at room temperature. The absorbance of samples was measured at 529 and 650 nm and anthocyanin content was determined by using the equation described in Sims & Gamon [37]. Results were expressed in micromoles per gram of dried weight.

Statistical and QTL analysis

A combined analysis of variance across organs and individual analyses of variance for each organ were made for the AA content measured ABTS, DPPH, FRAP assays and for phenolic, carotenoid and anthocyanin contents by using the procedure ANOVA of SAS v 9.2 [39]. Parental differences were analyzed one-tail "t" test by using PROC TTEST of SAS v 9.2 [39]. Simple correlation coefficients were computed with PROC CORR of SAS v 9.2 [39] for each trait.

The genetic map created by Iñiguez-Luy *et al.* [31] has 279 markers (SSRs and RFLPs) distributed along nine linkage groups (C1–C9) with a total distance of 891.4 cM and a marker density of 3.2 cM/marker. Quantitative trait locus mapping was carried out through a composite interval mapping method [40] by using PLABQTL [41]. Individual analyses were carried out for each trait and organ (leaves and flower buds). A likelihood odds (LOD) threshold was chosen for each trait in order to declare the putative QTL significant by following a permutation test, with N = 1000, and a critical alpha value of 25%. The confidence intervals were set to 95%. The analysis and cofactor election were carried out by following PLABQTL's recommendations, using an 'F-to-enter' and an 'F-to-delete' value of 7.

The proportion of phenotypic variance explained for a specific trait was determined by the adjusted coefficient of determination of regression (R^2) fitting a model which includes all detected QTLs [42]. Fivefold cross-validation of QTLs was performed by following the procedures described by Utz *et al.* [43]. The whole data set was randomly split into k = 5 data subsets. Four of these subsets were combined to form the estimation set (ES). The remaining subset formed the test set (TS), in which predictions derived from ES were tested for their validity by correlating predicted and observed data. We used 1,000 replicate CV/G runs. Estimates of medians and percentiles and the frequency of QTL detection in ES and TS were calculated over all replicated CV/G runs. The frequency of QTL detection gives us an estimation of the precision of QTL localization. The PLABQTL [41] software package was used for all calculations. Iñiguez-Luy *et al.* (2009) identified collinear genomic blocks between the BolTBDH mapping population and *A. thaliana* by using a synteny analysis. This information was employed in order to locate candidate genes which may directly account for QTLs in *B. oleracea*. By following this approach, we searched in the database TAIR (the *Arabidopsis* information resource http://www.arabidopsis.org) genes related to phenylpropanoid biosynthetic process metabolism (phenolic compounds and anthocyanins are synthetized following this pathway) and genes involved in the carotenoid biosynthetic process by including the words 'phenylpropanoid' and 'carotenoid' into the field 'description of the gene in TAIR. Twenty one genes related to phenylpropanoids and 24 genes related to carotenoids were found. We tried to locate these genes on the BolTBDH map by means of *in silico* mapping.

Results

Quantitative variation for methods measuring AA and the content of metabolites with AA

In this study AA in leaves and flower buds was determined by three ET methods: FRAP, DPPH and ABTS. The content of metabolites with AA (phenolics, anthocyanins and carotenoids) was also determined. We used two ET methods (DPPH and ABTS) where the scavenging was followed by monitoring the decrease in absorbance over time, which occurred due to the AA of the sample [44]. For the FRAP assay, the extract shows an increase of absorbance over time dependent on their AA [45]. A transgressive distribution was found for all traits in both organs (Fig. 1). Results obtained from each analysis are considered below.

FRAP, DPPH and ABTS assays

Mean values for the FRAP and DPPH methods in the population were lower than the corresponding values of ABTS assay in both organs (leaves and flower buds). In leaves, we found mean values of 18.36, 14.04 and 24.78 µmol Trolox g−1 DW in FRAP, DPPH and ABTS assays, respectively. In flower buds, we found values of 15.37, 12.51 and 25.16 µmol Trolox g−1 DW in FRAP, DPPH and ABTS assays, respectively (Table 1).

Population mean values between the two organs present highly significant differences for FRAP (F = 75.95, P = 0.0129) and DPPH (F = 65.09, P = 0.0150) methods.

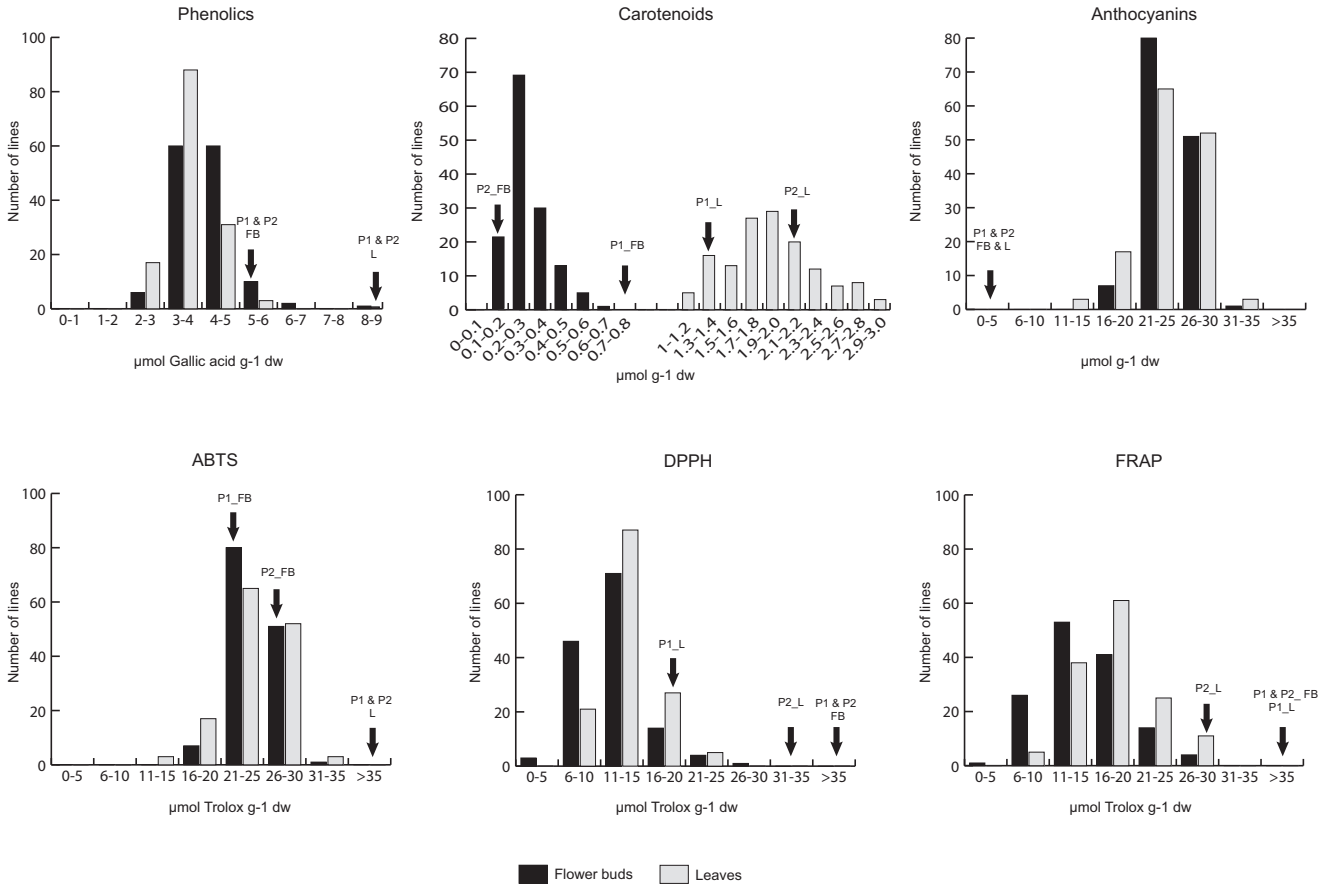

Figure 1. Distribution of the three metabolites with antioxidant activity, carotenoids, anthocyanins and phenolics and the three antioxidant assay methods, ABTS, DPPH and FRAP in the BolTBDH population. Arrows indicate values for the P1 (DH rapid cycling of Chinese kale TO1000DH3) and P2 (DH broccoli line 'Early Big') in the two organs under study, leaves (L) and flower buds (FB).
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Metabolites with AA: phenolic, anthocyanin and carotenoid content

Concerning the content of metabolites with AA, we found two different profiles. For the phenolics assay, population showed higher mean values in flower buds than in leaves (4.14 and 3.64 µmol gallic acid g−1 DW, respectively), although differences were not significant. However, both parental lines had higher phenolic content in leaves than in flower buds (Fig. 1).

Leaves of the mapping population had higher anthocyanin and carotenoid content (58.53 µmol g−1 DW and 1.98 µmol g−1 DW, respectively) compared to flower buds (13.2131 µmol g−1 DW and 0.28 µmol g−1 DW, respectively). Mean anthocyanin content of the population represents a strong increase compared to the values found in both parents. As other assays previously described, anthocyanins presented transgressive distributions for both organs (Fig. 1). In the case of carotenoid content, differences between both organs were highly significant (F = 80.44, P = 0.012). Correlation coefficients among methods measuring AA, phenolic

Table 1. Antioxidant activity of parents and population measured in leaves and flower buds with three different antioxidant assay systems and the content of three metabolites with antioxidant activity.

	Leaves			Flower buds		
Traits	**P1**	**P2**	**Population mean**	**P1**	**P2**	**Population mean**
ABTS (µmol Trolox g^{-1} DW)	42.06	44.89	24.78	21.13	30.94	25.16
DPPH (µmol Trolox g^{-1} DW)	20.20	34.18	14.04	50.65	47.84	12.51
FRAP (µmol Trolox g^{-1} DW)	48.17	56.27	18.36	59.40	28.71	15.37
PHENOLICS (µmol Gallic Acid g^{-1} DW)	8.02	8.91	3.64	5.55	5.54	4.14
ANTHOCYANINS (µmol g^{-1} DW)	0.03	0.67	58.53	0.04	0.13	13.31
CAROTENOIDS (µmol g^{-1} DW)	1.48	2.17	1.98	0.84	0.17	0.28

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and pigment contents in the BolTBDH population were made. Pairwise correlations between AA measured with three ET assays (FRAP, DPPH and ABTS) were positive and highly significant ($P \leq 0.01$) for both leaves and flower buds in the correlation analysis carried out with all lines of the mapping population. However, correlation coefficients were moderately low (Table 2). The highest correlations occurred between DPPH and FRAP assays for both organs. The correlation values were 0.486 in flower buds and 0.526 in leaves. On the other hand, correlation coefficients between the content of phenolic compounds and the three AA methods were positive and significant for both organs ($p \leq 0.01$). Significant correlations between the anthocyanin content with DPPH and ABTS were found in leaves. Correlation with DPPH was positive; however, correlation with ABTS was negative ($r = -0.339$, $p \leq 0.01$) (Table 2). Anthocyanin content was significantly and negatively correlated to ABTS assay (Table 2). Carotenoid content showed significant correlation coefficients with the AA measured with ABTS assay ($r = 0.140$, $p \leq 0.05$) in leaves, and significant and positive correlation coefficients with FRAP assay in flower buds ($r = 0.305$, $p \leq 0.01$). Furthermore, correlation between carotenoids and ABTS assay was negative and highly significant in flower buds ($r = -0.165$, $p \leq 0.01$) (Table 2).

QTL mapping for methods measuring AA, phenolic and pigment contents in the BolTBDH population

A total of 19 QTLs were detected for all traits. The number of QTLs by linkage group ranged between one in C9 and five in C3 (Fig. 2). For methods measuring AA, seven significant QTLs were found in leaves. The value of R^2 ranged between 9.8% for FRAP in C3 and 17.4% for DPPH in C4, respectively (Table 3). Three of these QTLs had a frequency of cross-validation higher than 50%. In flower buds, six significant QTLs were found. R^2 value varied between 9.8% for ABTS in C6 and 12.1% for FRAP content in C3, but only two of the QTLs had a frequency of cross-validation higher than 50%.

For the content of metabolites with AA, two significant QTLs for phenolic content were found in leaves. The value of R^2 ranged between 10.3 and10.4% in C7 and all of them had a frequency of cross-validation higher than 50%. Meanwhile, four significant QTLs were found in flower buds. The value of R^2 ranged between 9.9 and 12.6% for carotenoids in C5 and C9, respectively. Only one of these QTLs presents a frequency of cross-validation higher than 50%. One QTL for anthocyanin content was found on C3 in flower buds, from which a R^2 value of 10.9% and three QTLs for carotenoid content were found on C5, C8 and on C9. R^2 values varied between 9.9 and 12.6% (Table 3).

Based on the position of QTLs and taking into account their confidence interval, three genomic regions determined variability for different traits. The genomic region located on C3, in the interval from marker pW125dE to fito156c & pW133cH (AA-C3), determined variation for the three different methods measuring AA: FRAP in leaves and ABTS and DPPH in flower buds. A second genomic region on C7 from pW225aD to pW104aE (AA-C7) determined variation for the methods measuring AA (ABTS in leaves and FRAP in flower buds) and phenolic content in leaves. Alleles for increasing AA or phenolic content are given by P2 in both genomic regions on C3 and C7. A third genomic region on C5 (AA-C5), from pW209aH to Na10-F06b & fito132a, also determined variation for the methods measuring AA (DPPH in leaves and ABTS in flower buds) and carotenoid content in flower buds. In this case, alleles for increasing AA and carotenoid content are given by P1.

Genes related to phenylpropanoid biosynthesis were located by means of *in silico* mapping in the confidence interval of several QTLs (Table 4). However no gene related to carotenoid biosynthesis could be located.

Discussion

Quantitative variation for methods measuring AA and the content of metabolites with AA

Parents of the DH BolTBDH mapping population showed significant differences for the majority of the methods measuring AA and for the content of metabolites with AA in leaves and flower buds. BolTBDH population was found to be an ideal material in order to study QTLs for the traits under study in *Brassica* genus due to the differences between the two parents of this population. One parent (P2) is a broccoli 'Early Big' line, the *Brassica* crop with one of the highest AA [46], while the other parent (P1) is a DH rapid cycling line (TO1000DH3). Both parents are from different cultivars and as stated before, there is high variability for AA between different *Brassica* crops [16,26,34,47].

The total AA of a sample can be measured by using several methodologies [15–17,26]. The radical scavenging capacity of DH BolTBDH mapping population was measured by using three ET methods: ABTS FRAP and DPPH. The content of metabolites with AA like phenolics, anthocyanin and carotenoid was also measured. Some DH lines exhibited mean values of the traits falling between the values of the two parents, but others exhibited values which were extremely higher or lower than their parents. This phenomenon is referred to as transgressive segregation. Distributions of the methods measuring AA, phenolics and

Table 2. Correlation coefficients for leaves (above the diagonal) and flower buds (below the diagonal) between the three antioxidant assay methods and the content of three metabolites with antioxidant activity (n = 280).

Leaves/Flower buds	ABTS	FRAP	DPPH	PHENOLICS	ANTHOCYANS	CAROTENOIDS
ABTS	–	0.197**	0.267**	0.434**	−0.339**	0.140*
FRAP	0.189**	–	0.526**	0.151*	0.103	0.100
DPPH	0.389**	0.486**	–	0.250**	0.164**	0.051
PHENOLICS	0.633**	0.221**	0.227**	–	−0.110	0.086
ANTHOCYANINS	−0.130*	−0.027	−0.076	−0.100	–	−0.081
CAROTENOIDS	−0.165**	0.305**	0.005	−0.013	0.176**	–

* Significant at $p \leq 0.05$, and ** significant at $p \leq 0.01$. ABTS: 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); FRAP: ferric ion reducing antioxidant power assay; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

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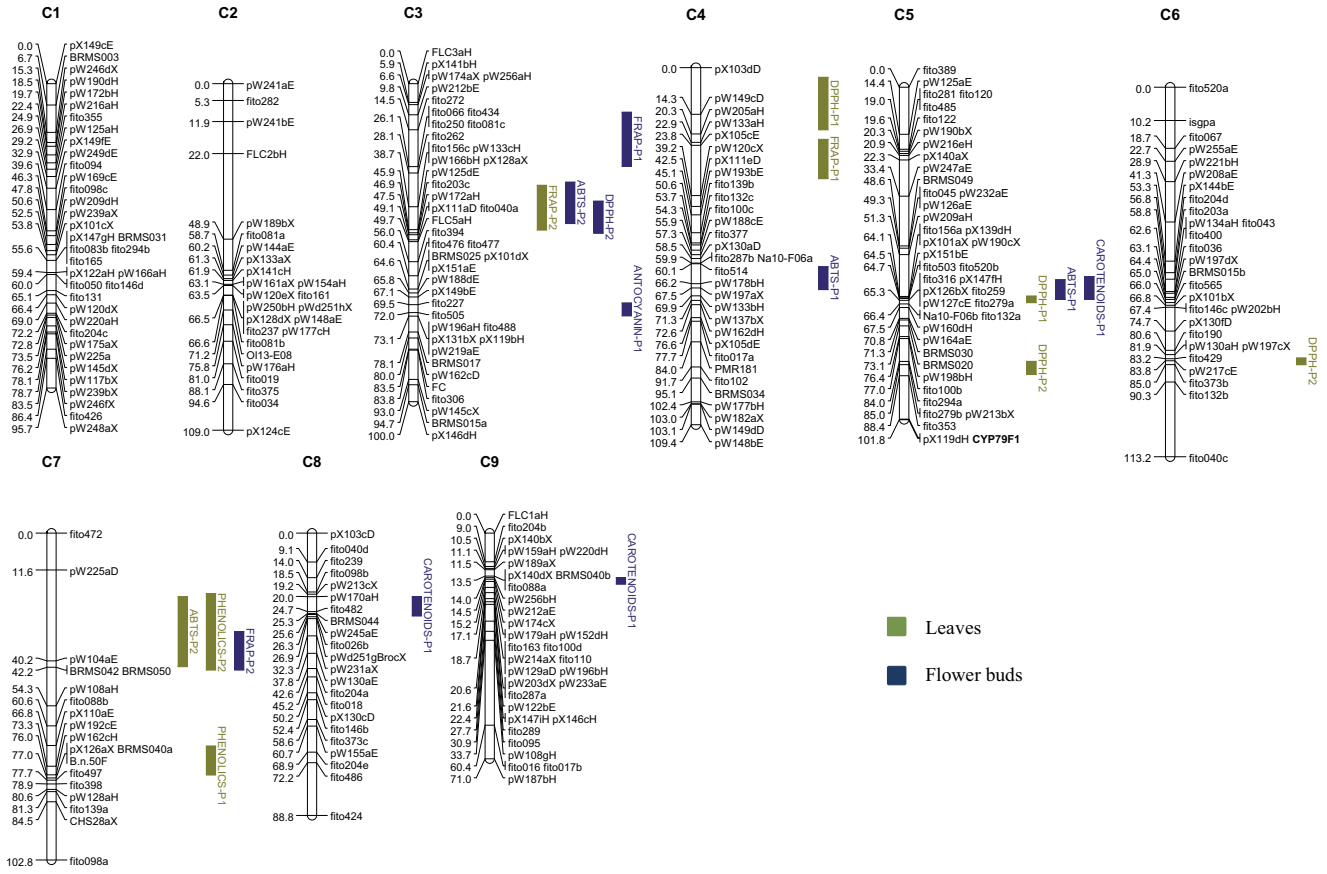

Figure 2. Framework map of DH population showing nineteen metabolic quantitative trait loci (QTL) for individual methods measuring AA. Linkage groups were labeled following the nomenclature of Iñiguez-Luy *et al.* [31]. Bars represent the LOD confidence interval of each QTL. QTLs are in different colors depending on the plant organ: leaves (green) and flower buds (blue). After the name of each QTL P1 indicates allele from, DH rapid cycling of Chinese kale (TO1000DH3) and P2 indicates allele from DH broccoli line 'Early Big'.
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pigment content were, in most cases, transgressive. The action of complementary genes may be the primary cause of transgression, although epistasis may also contribute [48]. Further studies could help to explain the transgressive segregation of the traits measured in this study. These studies could use other populations or add more molecular markers to our population.

Total AA varied considerably according to the organ under study. Generally speaking, leaves present higher AA and content of metabolites with AA than flower buds, as it was expected by their photosynthetic complex. This result is in agreement with Soengas *et al.* [16] and Llorach *et al.* [49], who measured the AA of heads and leaves of cauliflower, with the highest values found in leaves. Guo *et al.* [50] found similar values in both organs in broccoli and Soengas *et al.* [16] found that broccoli flower buds have higher AA than leaves. In broccoli and cauliflower, the organs which are consumed are the heads (flower buds) and the leaves surrounding the heads are treated as by-products. Our results show that leaves have more AA and content of metabolites with AA than heads. Therefore, consumption of broccoli by-products, which is one of the parents of the mapping population, could be an interesting option to include in the human diet.

Due to the characteristics of the methods analyzed, AA measured with FRAP and DPPH assays present lower values compared to that of ABTS assay. It is coincident with the results found by Gouveia *et al.* [51] in other species like *Andryala glandulosa*.

Correlation coefficients among methods measuring AA and the content of metabolites with AA

Significant correlation coefficients were found among the three methods measuring AA (FRAP, DPPH and ABTS) in the two organs under study, and ranged between 0.19 and 0.53. These correlations, although significant, were lower than others found in previous studies. Kusznierewicz *et al.* [17] found a correlation of 0.96 between ABTS and DPPH in white cabbage planted in different locations. Soengas *et al.* [16] found a correlation of 0.8 between DPPH and FRAP in extracts of different *Brassica* crops. Zhi *et* al. [18] found correlations ranging from 0.76 to 0.82 between the three cited methods analyzing different vegetables including broccoli. The material studied in our research is much closer genetically than the material studied in previously cited literature, since all the DH lines derive from a single cross. Clearly, correlations among ET methods depend on the material under study and based on our results, we recommend using more than one ET method in order to estimate the AA of a sample as suggested by Kurniereick *et al.* [17] and Gawlik-Dziki [52].

Significant correlations among the three methods measuring AA and the content of metabolites with AA were found in leaves and flower buds. Phenolic content was positively correlated with all the methods measuring AA. The correlation coefficient with ABTS showed the highest value in both organs. Several authors have found significant and high correlations (ranging from 0.7 to 1) between the AA measured with ABTS, DPPH and FRAP assays

Table 3. List of quantitative trait loci (QTL) for antioxidant activity and the content of metabolites with antioxidant activity in two plant organs under study, leaves and flower buds.

	Plant organ	Trait	Linkage group	Peak Position range (cM)	Left marker	Right marker	Lod theshold	LOD score	Frequency	Add	R^2%	adj R^2%
1	**Leaves**	**ABTS**	7	31 (20–42)	pW225aD	pW104aE	2.89	4.53	829	1.6354	14.2	12.5
2	**Leaves**	**DPPH**	4	12 (3–19)	pX103dD	pW149cD	2.85	5.45	972	−1.794	17.4	27.2
3			5	65 (64–66)	fito316, pX147fH, pX126bX, fito259, pW127cE & fito279a	Na10-F06b & fito132a		4.5	764	−2.112	14.1	
4			5	85 (84–88)	fito294a			3.83	605	2.202	12.2	
5			6	84 (83–85)	pW217cE	fito279b & pW213bX		3.64	249	3.455	11.6	
6	**Leaves**	**PHENOLICS**	7	34 (19–43)	pW225aD	pW104aE	3.02	3.23	278	0.294	10.4	10.5
7			7	73 (67–76)	pX110aE	pW192cE		3.2	213	−0.268	10.3	
8	**Leaves**	**FRAP**	3	41 (32–46)	fito156c, pW133cH, pW166bH & pX128aX	pW125dE	2.86	3.05	299	2.784	9.8	11
9			4	25 (22–34)	pX105cE	pW120cX		4.29	794	−2.518	13.5	
10	**Flower buds**	**ABTS**	3	38 (31–44)	fito262	fito156c, pW133cH, pW166bH & pX128aX	2.86	2.98	260	1.329	9.8	6.6
11			4	64 (61–68)	fito514	pW178bH		3.49	501	−2.441	11.4	
12			5	64 (59–65)	pW209aH	fito156a, pX139dH, pX101aX & pW190cX		3.28	348	−1.141	10.7	
13	**Flower buds**	**DPPH**	3	45 (37–47)	fito156c, pW133cH, pW166bH & pX128aX	pW125dE	2.86	3.11	48	1.402	10.2	1.3
14	**Flower buds**	**FRAP**	3	12 (9–26)	pW212bE	fito272	2.83	3.38	462	−2.325	11	6.8
15			7	40 (31–43)	pW225aD	pW104aE		3.72	631	2.538	12.1	
16	**Flower buds**	**Anthocyanin**	3	72 (69–73)	fito227	pW196aH, fito488, pX131bX, pX119bH & pW219aE	3.12	3.26	361	−4.458	10.9	0.2
17	**Flower buds**	**Carotenoid**	5	64 (58–65)	pW209aH	fito156a, pX139dH, pX101aX & pW190cX	2.93	2.94	226	−0.044	9.9	21.2
18			8	22 (20–26)	pW170aH	fito482		3.27	308	−0.047	10.9	
19			9	15 (14–16)	pW212aE	pW174cX		3.8	583	−0.049	12.6	

Additive effect was calculated as $(P_2–P_1)/2$; R^2% coefficient of determination of each QTL. Adj R^2%: determination coefficient of each trait.

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Table 4. List of phenylpropanoid biosynthesis candidate genes residing within the QTL confidence intervals according to organ and measurement method.

Plant organ	Trait	Markers in the confidence interval	Position in *Brassica oleracea* (cM) *	*Brassica oleracea* linkage group	Linkage group and position (bp) in *Arabidopsis thaliana**	Genes related to phenylpropanoid biosynthesis located in the interval of *Arabidopsis thaliana*	Candidate genes
Leaves	FRAP	fito156c	38.72	3	1(3530200–3530221)		
		pW133cH	38.72	3	2 (15610858–15610982)		
		pX128aX	38.72	3	5 (6804683–6804766)		
		pW125dE	45.85	3	5(2219504–2219693)		
						AT5G48930	HCT
Flower buds	ABTS	fito250	36.12	3	5(15688217–26579698)		
						AT5G48930	HCT
Flower buds	DPPH	fito156c	38.72	3	1(3530200–3530221)		
		pW133cH	38.72	3	2 (15610858–15610982)		
		pX128aX	38.72	3	5 (6804683–6804766)		
		pW125dE	45.85	3	5(2219504–2219693)		
						AT5G48930	HCT
Flower buds	FRAP	pW212bE	9.81	3	3(6427399–6427450)		
		fito066	26.12	3	4(6017387–6017408)		
						AT4G00040	CHS and SS
Leaves	FRAP	pX105cE	23.78	4	2(16117201–18117509)		
						AT2G40890	CYP98A3
Leaves	DPPH	pW217cE	83.82	6	1(14257280–18257453)		
						AT4G30210	
Leaves	ABTS	fito472	0	7	4(18268924–18269031)		
		pW104aE	40.25	7	1(126837519–26837557)		
						AT1G51680	4CL
Leaves	phenolics	fito472	0	7	4(18268924–18269031)		
		pW104aE	40.25	7	1(126837519–26837557)		
						AT1G51680	4CL
Flower buds	FRAP	fito472	0	7	4(18268924–18269031)		
		pW104aE	40.25	7	1(126837519–26837557)	AT1G51680	4CL

* Candidate gene found by means of *in silico* mapping in the *Arabidopsis thaliana* TAIR database. CHS and SS: Chalcone and stilbene synthase family protein; 4-CL: 4-coumarate: Co-A ligase 1, 2 or 3; HCT: hydroxycinnamoyltransferase enzyme; C4H: cinnamate 4-hydroxylase.

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and phenolic content measured with the Folin–Ciocalteu method in other *Brassica* crops (cabbages, broccoli and Brussels sprouts) [15,18,26,53,54]. These results confirm the hypothesis that phenolic compounds mainly account for the AA of *Brassica* extracts. In the review made by Podsedek *et al*. [26], it is pointed out that phenolic compounds have higher AA in *in vitro* experiments than vitamins and carotenoids.

Furthermore, positive and significant correlations between carotenoid content and methods measuring AA were found in flower buds (FRAP) and in leaves (ABTS) in this study. These correlations are smaller than those of phenolic compounds with AA. Our results confirm that carotenoids are metabolites which contribute to the AA of Brassica extracts. Krinsky *et al*. [55] described that phenolic and carotenoid content is positively correlated with AA. In the case of anthocyanins, our experiments do not show a clear relationship between their content and methods measuring AA.

QTL mapping for methods measuring AA and the content of metabolites with AA

Methods measuring AA on food extracts are extensively used by the scientific community in order to detect potential benefits for human health. Genetic variation for these traits is interesting from the breeder's points of view, since it could allow increasing the AA of *Brassica* foods by selection. As far as we know, no report of QTLs or genetic basis for methods measuring AA has been done before in any *Brassica* crop. This is also one of the first assays, which studies the genetic base of ET methods measuring AA in any crop. Only three recent pieces of research in rice [28], raspberry [29] and in lettuce [30] studied QTLs for total water AA, total phenolic content, anthocyanin and carotenoid content. Knowledge derived from this study can be utilized in order to search for genes underlying these traits.

Ten out of 19 QTLs determine AA or the content of metabolites with AA in only one of the two organs, thus indicating that the regulation of genes underlying several QTLs is organ-dependent. Seven QTLs determined variation for only one method measuring AA, thus indicating that the genetic basis regulation is partially dependent on the method. Genomic regions AA-C3, AA-C5 and AA-C7 determined variation for more than one ET method measuring AA. These genomic regions could be responsible for the significant correlations found between ET methods in this study.

The genomic region AA-C7 determines variation for methods measuring AA and phenolic compounds and the genomic region AA-C5 determines variation for methods measuring AA and carotenoid content. These finding supports the hypothesis that AA of the mapping population is related to phenolic compounds but also to carotenoid content. No QTLs related to methods measuring AA and anthocyanin content were found. Therefore, anthocyanins would not play a significant role in maintaining the AA of extracts in this population. The content of other compounds different from those under study could be responsible for the remaining QTLs, which control variation for methods measuring AA. The core reactions of phenylpropanoid metabolism involve several steps catalyzed by three key enzymes: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-Coumarate: CoA ligase (4CL) [56]. In *A. thaliana* there are 4CL different genes. This enzyme has a pivotal role in the biosynthesis of a plant's secondary compounds at the divergence point from general phenylpropanoid metabolism to several major branch pathways [57,58]. After *in silico* mapping analysis, 4CL-1 gene was located in the genomic region AA-C7 which controls AA measured as ABTS and FRAP and phenolic content. The hydroxycinnamoyltransferase enzyme (HCT) appears to be potentially implicated in the pathway both upstream and downstream of the 3-hydroxylation step and it is another key enzyme in phenylpropanoid biosynthesis. HCT enzyme catalyzes reactions both immediately preceding and following the insertion of the 3-hydroxyl group into the monolignol pathway [59–61] realised by the CYP98A3 (C3′H). HCT gene from *A. thaliana* was located by means of *in silico* mapping in the genomic region AA-C3, which controls AA measured with the three ET methods. C3′H gene was located in the interval of pX105cE to pW120cX on chromosome 4 where a QTL for AA measured with FRAP method was found. More candidate genes related to phenylpropanoid biosynthesis, along all the linkage group, were identified as it is the case of the chalcone and stilbene (CHS and SS) family protein which catalyzed the initial steps for flavonoid biosynthesis, route related with the phenylpropanoid biosynthesis [62]. More work is necessary in order to validate and confirm candidate genes for the QTLs found in this study.

Conclusions

No reports on the genetic basis of AA, and the content of metabolites with AA like phenolic, anthocyanin and carotenoid content have been documented before in *Brassica* crops. Results among methods measuring AA suggest that it is necessary to use more than one ET method in order to estimate AA, due to the fact that these methods present low significant correlations between them. Phenolic compounds and carotenoids are responsible for the AA of *Brassica* extracts.

Three genomic regions determined variation for more than one ET method measuring AA. QTL analysis confirms that AA of the mapping population is related to phenolic compounds but also to carotenoid content. It should be pointed out that the experiments have been carried on in one environment and under controlled conditions of temperature and light. Once the existence of QTLs for the traits under study has been proved, new experiments are going to be carried on in different environments to test the stability of the QTLs and the influence of environmental conditions. Several candidate genes related to phenylpropanoid biosynthesis are proposed for the QTLs found. These QTLs and the possible candidate genes identified through syntenic analysis with *A. thaliana* are the first step to understand the genetic basis of AA in the *Brassica* genus.

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Author Contributions

Conceived and designed the experiments: MEC PS. Performed the experiments: TS. Analyzed the data: TS PV. Wrote the paper: TS.

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Identification of Metabolic QTLs and Candidate Genes for Glucosinolate Synthesis in *Brassica oleracea* Leaves, Seeds and Flower Buds

Tamara Sotelo*, Pilar Soengas, Pablo Velasco, Víctor M. Rodríguez, María Elena Cartea

Group of Genetics, Breeding and Biochemistry of Brassicas, Department of Plant Genetics, Misión Biológica de Galicia, Spanish Council for Scientific Research (MBG-CSIC), Pontevedra, Spain

Abstract

Glucosinolates are major secondary metabolites found in the *Brassicaceae* family. These compounds play an essential role in plant defense against biotic and abiotic stresses, but more interestingly they have beneficial effects on human health. We performed a genetic analysis in order to identify the genome regions regulating glucosinolates biosynthesis in a DH mapping population of *Brassica oleracea*. In order to obtain a general overview of regulation in the whole plant, analyses were performed in the three major organs where glucosinolates are synthesized (leaves, seeds and flower buds). Eighty two significant QTLs were detected, which explained a broad range of variability in terms of individual and total glucosinolate (GSL) content. A meta-analysis rendered eighteen consensus QTLs. Thirteen of them regulated more than one glucosinolate and its content. In spite of the considerable variability of glucosinolate content and profiles across the organ, some of these consensus QTLs were identified in more than one tissue. Consensus QTLs control the GSL content by interacting epistatically in complex networks. Based on *in silico* analysis within the *B. oleracea* genome along with synteny with *Arabidopsis*, we propose seven major candidate loci that regulate GSL biosynthesis in the *Brassicaceae* family. Three of these loci control the content of aliphatic GSL and four of them control the content of indolic glucosinolates. GSL-ALK plays a central role in determining aliphatic GSL variation directly and by interacting epistatically with other loci, thus suggesting its regulatory effect.

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* E-mail: tsotelo@mbg.csic.es

Introduction

The *Brassica* genus includes six agricultural important species which are grown in many countries, and important oil, condiment and vegetable crops. *Brassica* vegetables like broccoli, cabbage, Chinese cabbage, turnip greens and leaf rape, among others, are consumed throughout the world. FAO Statistics (FAOStat 2011) show that the production of cauliflower, broccoli, kales and other crucifers was 8.2% of the total vegetable production of the world in 2011. The most consumed crop of this genus in Europe and the USA is *Brassica oleracea*. This species includes cabbages, kales, broccoli and cauliflower, among others.

Glucosinolates (GSLs) are the major class of secondary metabolites found in the Brassicaceae falily, including the *Brassica* genus. The hydrolytic breakdown products of GSLs (especially isothiocyanates) have beneficial effects on human health, such as cytotoxic and apoptotic effects in damaged cells, thus preventing cancer in humans and reducing the risk for degenerative diseases [1–3]. They also enhance plant protection to abiotic and biotic stresses [4]. GSLs could exhibit certain adverse effects. For example, progoitrin can cause goiter in animals [5], which provoked the deliberate reduction of GSL levels in *B. napus* in the past. However, there is no evidence of any goitrogenic effect coming from *Brassica* consumption in humans [6]. Currently, efforts are concentrated on increasing the level of health promoting GSLs in *Brassica* crops. For example Sarikamis *et al.* [7] selected broccoli for higher levels of 3-methylsulphinylpropyl (GIB) and 4-methylsulphinylbutyl (GRA), which are the precursors of the isothiocyanates called iberin and sulforaphane, respectively. The beneficial effects of both isothiocyanates on human health are well known, having an influence on carcinogenesis during the initiation and promotion phases of cancer development [8]. Knowledge on the genetics underlying the synthesis and accumulation of GSLs in *Brassica* crops is an important tool for designing appropriate strategies in order to increase the content of those GSLs related to human health and plant protection.

GSLs are divided into three different classes according to the amino acid precursor in biosynthesis: (1) aliphatic GSLs derived from alanine (Ala), leucine (Leu), isoleucine (Ileu), valine (Val), and methionine (Met); (2) aromatic GSLs derived from phenylalanine (Phe) and tyrosine (Tyr) and (3) indolic GSLs derived from tryptophan (Trp) [9]. In *Arabidopsis thaliana* and *Brassica* crops, most GSLs are synthesized from Met. GSL biosynthesis is a tripartite pathway involving three independent steps (Fig. 1A): (i) side chain elongation of some precursor amino acids such as Met and Phe, by adding one or several methylene groups. Chain elongation is

carried out by methylthioalkylmalate synthase enzymes (MAM). (ii) Development of the core structure, which includes several steps: aldoxime formation catalyzed by the CYP79 family of cytochromes P450; aldoxime oxidation by the CYP83 family; thiohydroximic acid formation by conjugation to an S donor and after C-S bond cleavage; desulfoGLS formation by S-glucosyltransferase (S-GT); and GSL formation by sulfotransferase. (iii) Secondary modification of the amino acid side chain which includes oxidation, hydroxylation, methoxylation, desaturation, sulfation, and glycosylation [10,11].

To date, major genes and transcription factors involved in the three steps of GSL biosynthesis have been identified and characterized in the model plant, *A. thaliana*. Based on *A. thaliana* homology, three loci were identified in *B. oleracea* and cloned [12–14]: two loci responsible for the elongation of the side chain of aliphatic GSLs named BoGSL-ELONG and BoGSL-PRO (homologous to MAM-1 and MAM-2 genes, respectively of *Arabidopsis*) and one locus responsible for side the chain desaturation and production of an alkenyl GSL named BoGSL-ALK (homologous to AOP2 gene of *Arabidopsis*). Afterwards, these loci, plus genes BoCS-lyase, BoGS-OH and BoCYP79F1, were mapped [15]. However, genes responsible for other steps of the metabolic pathway remain undiscovered. Identification of metabolic QTLs (QTLs) is essential for the understanding of the quantitative genetic control of secondary metabolites and it is an early step to identify the genes underlying trait variation. The high co-linearity between *A. thaliana* and *Brassica* species can be used in order to identify candidate genes underlying QTLs that affect GSL content. In addition to identifying structural and accumulation QTLs, it is important to determine the extent of epistatic interactions between loci which may play an important role in determining variability for GSL content.

The accumulation and profile of GSLs in plants are highly dependent on the genotype, although it is also affected by environmental and developmental factors. In *Arabidopsis*, GSL profiles have been systematically monitored during plant development and vary significantly among tissues and organs [16–19]. In *B. oleracea*, developmental stages and the type of tissues may modify the type of GSLs and its levels [20,21]. Currently, little is known about the genetics of GSL content within the plant ontogeny. For this reason, it is necessary to develop a better understanding of the genetics underlying GSL biosynthesis and accumulation in different tissues in *B. oleracea*.

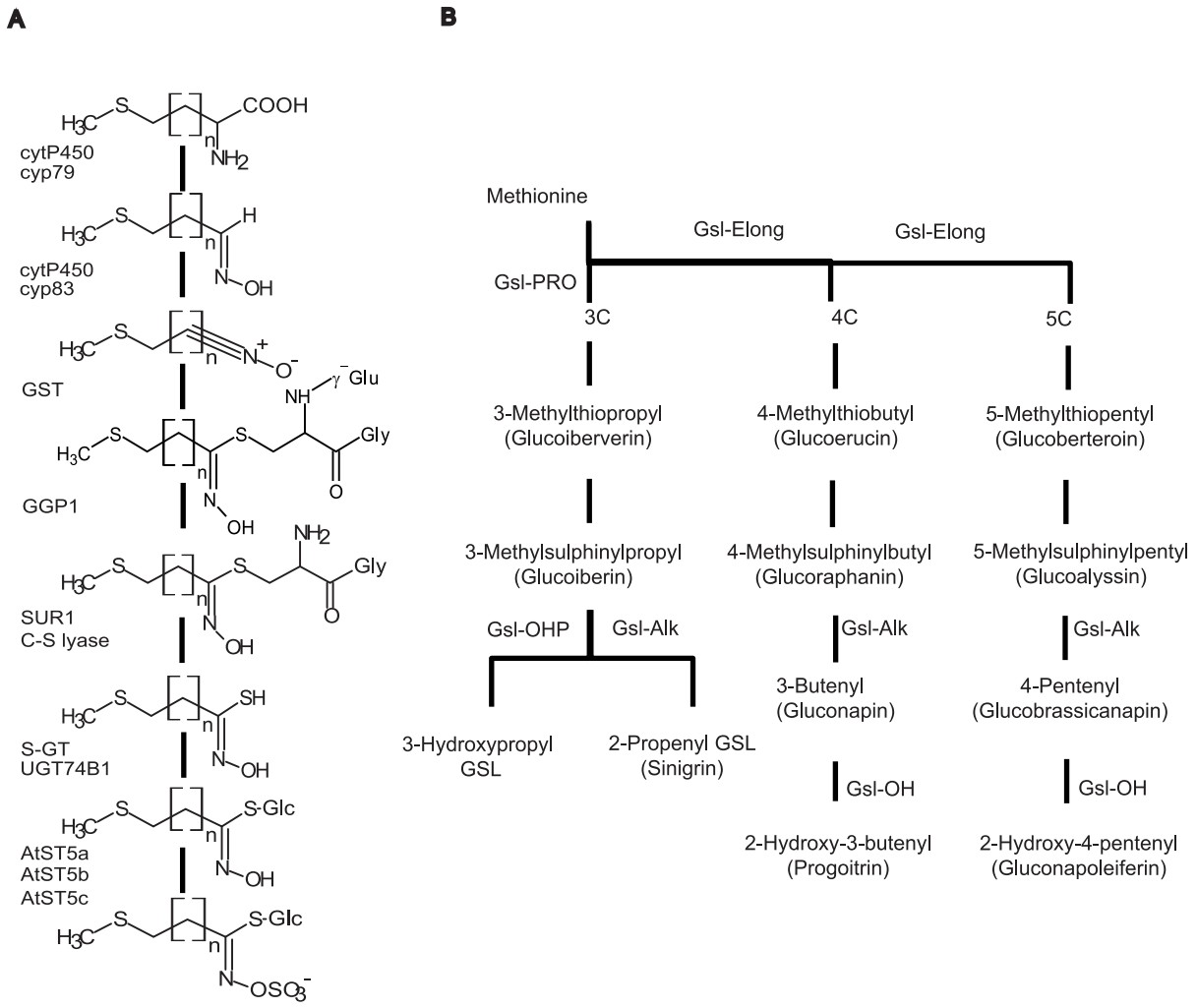

Figure 1. Formation of the core structure of the three major groups of glucosinolates in *A.thaliana*, including the genes controlling this process [11,43]. (A). A biochemical genetic model of the biosynthesis of aliphatic glucosinolates in *Brassicaceae* including the major genes controlling this process [57] (B).
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In the present study we identify QTLs for GSL composition and accumulation in *B. oleracea* leaves, flower buds and seeds in a double haploid (DH) population. We also perform a comparative genomic analysis based on *A. thaliana-B. oleracea* synteny in order to find candidate genes underlying QTL variation. Epistatic relationships among QTLs are also described. This information may increase the understanding on the quantitative genetic control of these traits and it is useful in order to identify genes controlling GSLs in *B. oleracea*.

Materials and Methods

Plant material and growing environments

A double haploid (DH) mapping population (BolTBDH) was employed in this work. The population was created from an F_1 individual, from a cross between a DH rapid cycling of Chinese kale (TO1000DH3, P_1) and a DH broccoli line 'Early Big' (P_2) [22]. TO1000DH3 is the reference genome for the *B. oleracea* sequencing project. Firstly, parents and 155 DH lines were grown and selfed in the greenhouse in 2010 under: 16 h of daylight and a temperature of 24±2°C; 8 h of darkness having 18±2°C at night; and a relative humidity of 55% in order to obtain enough seed in the same environmental conditions. Selfing was carried out by bagging each individual plant inside a microperforated polyethylene bags. Five bulks of 10 mg of seed for each line were prepared for GSL analysis with the seeds obtained. In 2011 (from September to November), seeds from parents and 155 DH lines were sown with the same photoperiod and temperature as in 2010. Plants were sown in a completely randomized experiment with two replications and 4 plants per replication and DH line.

From each line, leave samples were taken at the 4 leaf stage and flower buds were taken differentially depending on the flowering time of each plant. One bulk was taken from each replication by mixing the four samples of leaves and flower buds. Samples were immediately frozen in liquid N_2, transferred to the laboratory and conserved at –80°C until processing. All samples were lyophilized (BETA 2–8 LD plus, Christ) during 72 h. The dried material was powdered by using an IKA-A10 (IKA-Werke GmbH & Co.KG) mill, and the fine powder was used for GSL extraction.

GSL identification and quantification

Sample extraction and desulfation were performed according to Kliebenstein *et al.* [23] with minor modifications. Three microliters of the desulfo-GSL extract for seeds and 5 µl for leaves and flower buds were used in order to identify and quantify GSLs. Chromatographic analyses were carried out on an Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The UHPLC column was a C18 Atlantis T3 waters column (3 µm particle size, 2.1×100 mm i.d.) protected with a C18 guard cartridge. The oven temperature was set at 30°C. Compounds were detected at 229 nm and were separated by using the following method in aqueous acetonitrile, with a flow of 0.8 mL min^{-1}: 1.5 minutes at 100%H_2O; a 11 min gradient from 0% to 25% (v/v) acetonitrile; 1.5 min at 25% (v/v) acetonitrile; a minute gradient from 25% to 0% (v/v) acetonitrile; and a final 3 min at 100% H_2O. Data were recorded on a computer with the LabSolutions software (Shimadzu). Specific GSLs were identified by comparing retention times with standards and by UV absorption spectra.

GSLs were quantified at 229 nm by using sinigrin (SIN, sinigrin monohydrate from Phytoplan, Diehm& Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS, glucobrassicin potassium salt monohydrate, from Phytoplan, Diehm& Neuberger GmbH, Heidelberg, Germany) as external standards and expressed in µmol g^{-1} dry weight (DW). Calibration equations were made with, at least, five data points, from 0.34 to 1.7 nmol for sinigrin and from 0.28 to 1.4 nmol for glucobrassicin. The average regression equations for sinigrin and glucobrassicin were $y = 148818\times$ ($R^2 = 0.99$) and $y = 263822\times$($R^2 = 0.99$), respectively.

Statistical analysis

A combined analysis of variance across organs and individual analyses of variance for each organ were made for individual and total GSL. Lines and organs were considered as fixed factors and replications were considered as random factors. Analysis of variance was performed with the PROC GLM of SAS [24].

The genetic map employed for the QTL analysis was created by Iñiguez-Luy *et al.* [22] having 279 markers (SSRs and RFLPs) distributed along nine linkage groups (C1-C9) with a total distance of 891.4 cM and a marker density of 3.2 cM/marker. Eight primer pairs described by Gao *et al.* [15] amplifying loci BoGSL-ELONG, BoGSL-ALK, BoGSL-PROa, BoGSL-PRO-b, BoCS-lyase, BoGS-OH, BoCYP79F1 and BoS-GT from *B. oleracea* were screened in parent DH lines. Besides, SSRs Gi12 Hasan *et al.* [25] and Ol12-D05 [26] were screened in parental DH lines. SSRs Gi12 and Ol12-D05 map in both sides of ATR1 gene of *A. thaliana* in chromosome 5 [25]. Amplifications were performed by following Gao *et al.* [15] and electrophoresis was carried out in 1% agarose gels and capillary electrophoresis system (CEQ 8000 Beckman, Coulter). Polymorphic markers were then screened in the BolTBDH mapping population, scored and assigned to linkage groups with JoinMap 3.0 sofware [27]. The threshold for assigning markers to linkage groups was a LOD score between 5 and 8.

Quantitative trait locus mapping was carried out thanks to a composite interval mapping method [28] by using the PLABQTL program [29]. In each organ (leaves, flower buds and seeds), analyses were carried out on each individual GSL and for each GSL type (aliphatic, indolic and aromatic) as well as on the total GSLs. A likelihood odds (LOD) threshold of 3.2 was chosen in order to declare a putative QTL significant by following the method described by Van Ooijen [30]. The confidence intervals were set at 95%. The analysis and cofactor election were carried out by following PLABQTL's recommendations, by using an 'F-to-enter' and an 'F-to-delete' value of 7.

The proportion of phenotypic variance explained for a specific trait was determined by the adjusted coefficient of determination of regression (R^2) fitting a model including all detected QTLs [31]. Fivefold cross-validation of QTLs was performed by following the procedures described by Utz *et al.* [32]. The frequency of QTL detection gives us an estimation of the precision of QTL localization.

Significant QTLs for individual GSLs were integrated by using a QTL meta-analysis with BioMercator 2.1 software in order to give consensus QTLs [33]. An Akaike-type statistical criterion (AIC value) indicated the model which best fitted the data, including the number and the consensus QTLs positions. The aim of performing a meta-analysis was to find if a genomic region could determine the GSL content of different GSLs and if the same QTL was present in the three organs under study.

Iñiguez-Luy *et al.* [22] identified collinear genomic blocks between the BolTBDH mapping population and *A. thaliana* by using a synteny analysis. This information was employed in order to identify candidate genes that may directly account for GSL QTLs in *B. oleracea*. In following this approach, we tried to locate 46 genes involved in GSL metabolism in *A. thaliana* which were

Table 1. Glucosinolate (GSL) profiles and concentrations (μmol g-1dw) of parents and mean and range of the DH population.

	Leaves				Flower buds				Seeds			
GSL	P_1	P_2	Population mean (range)	Population %	P_1	P_2	Population mean (range)	Population %	P_1	P_2	Population mean (range)	Population %
GIV	-	-	-	-	-	-	-	-	0.53	0.00	1.63 (0–6.81)	1.39
GIB	0.00	0.00	0.29 (0–1.10)	5.33	0.00	0.00	0.89 (0–3.40)	6.60	1.04	0.00	6.06 (0–41.20)	5.14
SIN	2.42	0.00	0.44 (0–1.57)	8.02	1.57	0.00	1.22 (0–4.51)	9.04	42.32	0.00	8.15 (0–46.82)	6.91
GER	-	-	-	-	0.00	0.20	0.18 (0–0.50)	1.30	0.54	7.27	8.25 (0.27–34.54)	6.99
GRA	0.00	0.45	0.97 (0–6.65)	17.63	0.21	5.14	3.64 (0.15–17.35)	26.93	0.72	21.69	22.62 (0.48–74.14)	19.17
GNA	3.56	0.00	0.86 (0–6.38)	15.64	3.09	0.00	3.12 (0–17.12)	23.02	77.31	0.00	44.50 (0–138.40)	37.72
PRO	0.00	0.00	0.56 (0–2.77)	10.19	0.51	0.12	1.12 (0–13.22)	8.28	0.94	0.00	20.45 (0–129.80)	17.33
ALY	-	-	-	-	-	-	-	-	0.00	0.00	0.25 (0–2.38)	0.22
OHGBS	0.00	0.00	0.034 (0–0.36)	0.62	0.00	0.09	0.13 (0–0.41)	0.98	4.80	1.66	4.34 (1.81–10.20)	3.68
GBS	0.68	1.30	1.02 (0.005–3.24)	18.50	0.35	0.52	0.97 (0.14–3.87)	7.17	0.00	0.40	0.75 (0–5.37)	0.64
NeoGBS	1.72	2.34	1.14 (0.069–6.39)	20.63	0.59	1.06	1.86 (0.13–11.84)	13.78	0.53	0.37	0.50 (0–1.70)	0.43
GNT	0.19	0.79	0.19 (0–0.79)	3.44	0.18	0.86	0.39 (0–1.15)	2.90	0.38	0.21	0.42 (0–1.39)	0.36
Aliphatic	5.97	0.65	1.58 (0–6.97)	40.20	5.38	5.29	6.63 (0.59–20.98)	67.70	123.70	28.97	77.78 (30.38–157.15)	93.34
Indolic	2.40	3.65	2.17 (0.09–8.47)	55.21	0.94	1.68	2.88 (0.46–12.14)	28.57	5.33	2.44	5.29 (2.12–10.29)	6.36
Aromatic	0.19	0.79	0.19 (0–0.79)	4.22	0.18	0.86	0.39 (0–1.15)	3.86	0.38	0.21	0.42 (0–1.39)	0.50
Total	8.56	5.09	4.01 (0.12–13.20)	100.00	6.50	7.99	10.13 (1.47–24.56)	100.00	129.41	31.61	83.33 (36.23–160.29)	100.00

P_1, DH rapid cycling of Chinese kale (TO1000DH3); P_2DH broccoli line 'Early Big'; Aliphatic glucosinolates: GIV, Glucoiberverin; GIB, Glucoiberin; SIN, Sinigrin; GER, Glucoerucin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; ALY, Glucoalyssin; GBN, Glucobrassicanapin; Indolic glucosinolates: OHGBS, 4-hydroxyglucobrassicin; GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin; Aromatic glucosinolate: GNT, Gluconasturtiin.

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obtained from TAIR (The *Arabidopsis* Information Resource) on the BolTBDH map by *in silico* mapping.

Epistatic interaction analysis among QTLs was performed by using the R/qtl package of the R software [34].

Results

Phenotypic variation in GSL content

Twelve GSLs, belonging to three chemical classes, were detected in the BolTBDH population (Table 1). Eight GSL were aliphatic, three of them belonging to the 3C group: 3-methylthiopropyl (GIV), 3-methylsulfinylpropyl (GIB) and 2-propenyl (SIN); four belonging to the 4C group: 4-methylthiobutyl (GER), 4-methylsulfinylbutyl (GRA), 3-butenyl (GNA) and 2-hydroxy-3-butenyl (PRO); and one belonging to the 5C group: 5-methylsulfinylpentyl (ALY). Three indolic GSLs: 4-hydroxy-3-indolylmethyl (OHGBS), 3-indolylmethyl (GBS); and 1-methoxy-3-indolylmethyl (NeoGBS), and one aromatic GSL, 2-phenylethyl (GNT), were also detected.

Different GSL profiles were detected in the parental lines. The following aliphatic GSLs were found in P_1 (TO1000DH3) in different organs: GIV, GIB, SIN GER, GRA, GNA, and PRO. Aliphatic GER and GRA and PRO were detected in P_2 ('Early Big' broccoli) meantime aliphatic ALY was found in the mapping population but it was not detected in its parents. Therefore, 3C and 4C GSLs were found in P_1, while only 4C GSLs were found in P_2. Alkenyl GSLs (SIN, GNA and PRO) were found in P_1 but not in P_2 (only trace amounts of PRO in flower buds) (Table 1).

The GSL profile of the mapping population varied depending on the organ. In leaves, 55.2% of GSLs were indolic and 40.2% of GSLs were aliphatic, being NeoGBS and GRA the major GSLs respectively. In seeds, 93.3% of total GSLs were aliphatic, and GRA, GNA and PRO were the major GSLs. The GSL profile of flower buds was intermediate among leaves and seeds as 67.7% of total GSLs were aliphatic and 28.6% were indolic. GRA, GNA and NeoGBS were the major GSLs in this organ. GIV and ALY were exclusively found in seeds, meanwhile GER was only found in flower buds and seeds (Table 1).

Aliphatic GSL content in P_1 was higher than that found in P_2 in the three organs analyzed (Table 1). SIN and GNA were the major aliphatic GSLs found in the three organs for P_1. In contrast GRA was the major GSL in P_2 in the three organs. Regarding indolic GSLs, GBS and NeoGBS were found as the most abundant in both parents in both leaves and flower buds, while OHGBS was the major GSL found in seeds. Indolic GSL content was higher in P_2 compared to P_1 in both leaves and flower buds.Total GSL content in P_1 was higher than that found in P_2 leaves and seeds (Table 1).

In the mapping population, the content of individual GSLs as well as the content of aliphatic, indolic and total GSLs showed continuous distributions. Extreme phenotypes were found for all traits, with the exception of GNT in leaves, compared to phenotypes observed in parent lines (Table 1). For example, extreme mean values of some individual GSL content in the mapping population are far beyond the content of any of the parents. For instance, GRA content in seeds was 0.72 μmol g^{-1}dw

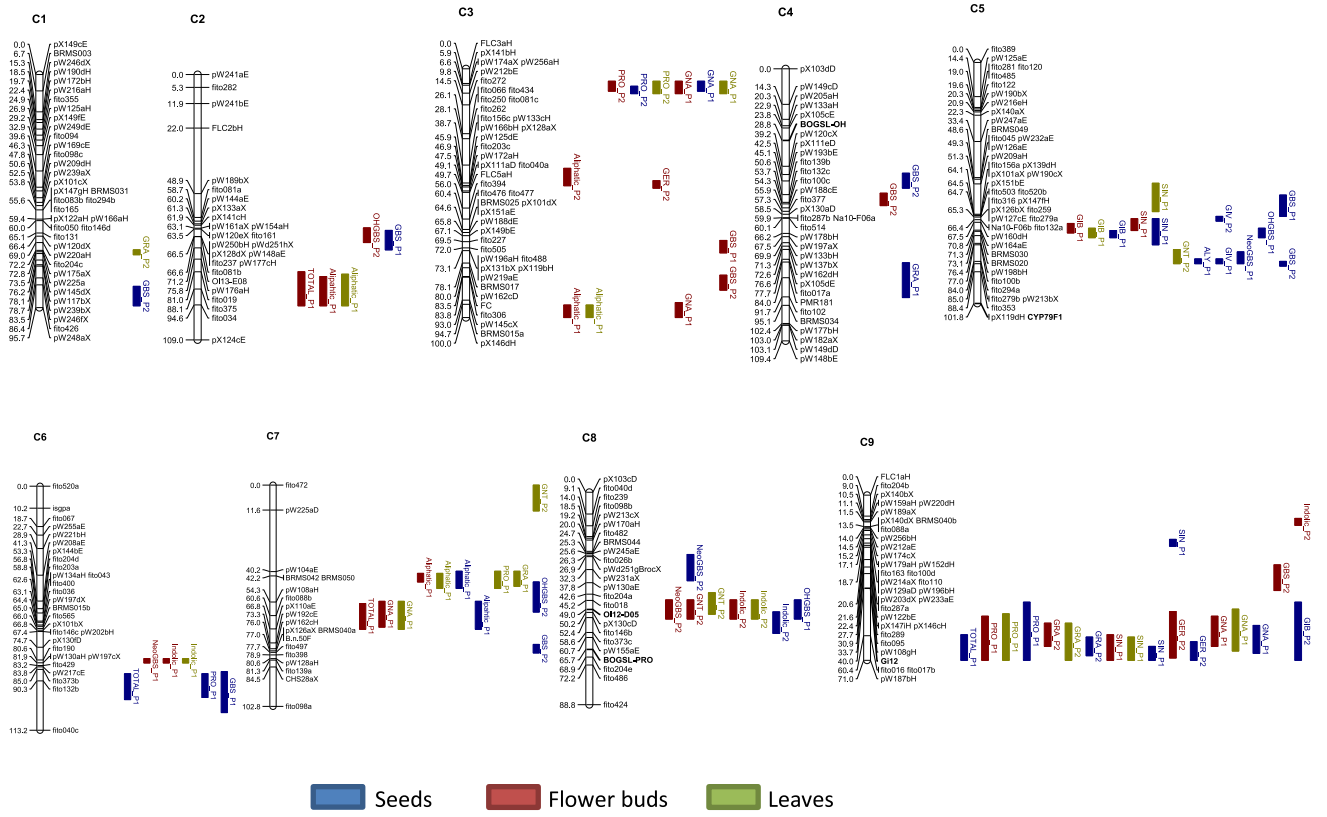

Figure 2. Framework map of DH population showing eighty-two metabolic quantitative trait loci (QTL) for individual GSLs and sums of GSLs. Linkage groups were labeled by following the nomenclature of Iñiguez-Luy *et al.* [22]. Bars represent the LOD confidence interval of each QTL. QTLs are in different colors depending on the plant organ: leaves (green), flower buds (red) and seeds (blue). After the name of each QTL, -P_1 indicates allele from DH rapid cycling of Chinese kale (TO1000DH3) and -P_2 indicates allele from DH broccoli line 'Early Big'.
doi:10.1371/journal.pone.0091428.g002

Table 2. Position and characteristics of consensus QTLs found in BolTBDH mapping population.

				Aliphatic																		
				3C			4C				5C			Indolic				Aromatic	Total	Plant organ		
LG	No	Peak position (cM)	Confidence interval (cM)	GIV	GIB	SIN	GER	GRA	GNA	PRO	ALY	GBN	Sum of aliphatic GSLs	OHGBS	NeoGBS	GBS	Sum of indolic GSLs	GNT	Total GSL	Seeds	Leaves	Flower buds
2	2.1	67.55	62.3–78.9											x		x				x		x
	2.2	89.74	83.7–95.7										x						x		x	x
3	3.1	7.09	4.9–9.3						x	x										x	x	x
	3.2	46.6	36.4–56.8				x						x									x
	3.3	79.1	69.0–89.2													x						x
	3.4	99.3	92.6–105.9						x				x								x	x
4	4.1	47.3	37.1–57.4													x				x		x
	4.2	84.0	70.9–97.0					x												x		
5	5.1	70.2	68.3–72.0	x	x	x								x		x				x	x	x
	5.2	84.1	81.9–86.3	x							x				x	x		x		x	x	
6	6.1	86.8	81.9–91.7							x					x	x	x		x	x	x	x
7	7.1	2.0	0–17.8															x			x	
	7.2	42.9	40.3–45.4					x		x			x							x	x	x
	7.3	56.4	52.3–60.5						x				x	x					x	x	x	x
	7.4	76.0	67.7–84.2													x				x		
8	8.1	45.8	41.5–50.1											x	x		x	x		x	x	x
9	9.1	27.0	20.7–33.3			x										x	x			x		x
	9.2	64.9	63.2–66.6		x	x	x	x	x	x									x	x	x	x

Aliphatic glucosinolates: GIV, Glucoiberverin; GIB,Glucoiberin; SIN, Sinigrin; GER, Glucoerucin; GRA,Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; ALY, Glucoalyssin; GBN, Glucobrassicanapin. Indolic glucosinolates: OHGBS, 4-hydroxyglucobrassicin; GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin. Aromatic glucosinolate: GNT, Gluconasturtiin.

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in P_1 and 21.69 μmol g^{-1}dw in P_2. The average GRA content in the mapping population was 22.62 μmol g^{-1}dw and ranged from 0.48 to 74.14 μmol g^{-1}dw (Table 1). Total GSL content in the different organs varied nearly 18-fold within the mapping population. The average content of total GSLs was 4.01 μmol g^{-1}dw in leaves, 10.13 μmol g^{-1}dw in flower buds and 83.3 μmol g^{-1}dw in seeds (Table 1).

Analysis of variance

Significant organ x line interactions were found for all traits, therefore individual analyses were carried out by organ. The source of variation due to lines was highly significant for the most traits, except ALY and OHGBS in leaves and GIV and NeoGBS in seeds. The source of variation due to replications was in most cases non significant (data not shown).

QTL analysis

Three out of eight primer pairs designed by Gao *et al.* [15] were polymorphic in of the mapping population's parents. These markers could be mapped and located in three different linkage groups. BoGSL-OH mapped on C4 (28.8 cM), BoCYP79F1 mapped on C5 (102 cM) and BoGSL-PROb mapped on C8 (66 cM). SSRs OL12-D05 and Gi12 were also polymorphic and they mapped on C8 (49 cM) and C9 (40 cM), respectively. QTL analyses were carried out with 279 markers designed by Iñiguez – Luy and the five newly mapped primer pairs. No significant QTL was detected in any of the map positions where BoGSL-OH, BoCYP79F1 and BoGSL-PROb were located (Fig. 2).

Eighty-two significant QTLs were detected being spread all over the 9 linkage groups of *B. oleracea*. The number of QTLs by linkage group ranged between two in C1 and 19 in C9 (Fig. 2). Twenty significant QTLs were found in leaves. The value of R^2 ranged between 10.3% for GNA in C7 and 34.3% for the sum of aliphatic GSLs in C7 (Table S1). Half of QTLs had a frequency of cross-validation higher than 50%. Twenty-nine significant QTLs were detected in flower buds. R^2 value ranged between 10.4% for the sum of aliphatic GSLs in C3 and 49.7% for the sum of aliphatic GSLs in C9, respectively. Eighteen QTLs had a frequency of cross-validation higher than 50%. Thirty-three significant QTLs were found in seeds. R^2 value varied between 10.3% for the sum of indolic GSLs in C6 and 49.4% for ALY in C5. Twenty-eight QTLs had a frequency of cross-validation higher than 50%.

Consensus QTLs

Based on the position of the QTLs and taking into account their confidence interval, a meta-analysis in order to render consensus QTLs for GSL concentration was carried out. Eighteen consensus QTLs were detected (Table 2). Fourteen consensus QTLs were present in seeds, 12 QTLs in leaves and 14 QTLs in flower buds. Seven QTLs were common to flower buds, leaves and seeds; three QTLs were exclusively found in leaves, two QTLs were exclusively found in flower buds and other two QTLs were exclusive found in seeds. In order to make the discussion clearer, results regarding consensus QTLs are going to be presented according to each chemical GSL class.

Aliphatic GSLs

Located in C3, consensus QTL-3.1 controls the content of PRO and GNA in the three organs (Table 2). Alleles for increasing PRO content are given by P_1, while alleles for increasing GNA content are given by P_2 (Fig. 2). Consensus QTL-5.1, located in C5, controls the content of GIB and SIN in the three organs. Alleles for increasing the content of both GSLs are given by P_1. In C9, consensus QTL-9.2, which controls the content of PRO, GNA, GRA, GER (4C-GSL) and SIN, and GIB (3C-GSL) in the three organs, was located. Alleles for synthesis of PRO, SIN and GNA are given by P_1, while alleles for increasing the content of GRA, GER and GIB are given by P_2 (Fig. 2). Other QTLs which control aliphatic GSL content exclusively are QTL-1.1, QTL-2.2, QTL-3.1, QTL-3.2, QTL-3.4, QTL-4.2 and QTL-7.2.

Indolic and aromatic GSLs

Several consensus QTLs only controlled the indolic GSL content. QTL-1.2, QTL-3.3, QTL-4.1 and QTL7.4 determined the GBS content in seeds and flower buds (Table 2). Alleles for increasing the content of GBS are given by P_2 in all these QTLs except for QTL-3.3, where alleles came from both parents. Consensus QTL-2.1 determines the content of OHGBS and GBS in seeds and flower buds. The allele for increasing OHGBS is given by P_2 in flower buds, while the allele for increasing GBS content is given by P_1. Consensus QTL-8.1 determines the OHGBS, NeoGBS and total indolic GSL content in the three organs. Besides, this QTL also controls the content of the aromatic GNT. Other QTLs for GNT content are QTL5.2 and QTL7.1. The genomic regions QTL-1.2, QTL-2.2 and QTL-7.4 are collinear with genomic regions of *A. thaliana* in chromosomes 4, 5 and 2. In these regions, genes CYP83B1, CYP81F2 and CYP79B3 from *A. thaliana* were found by means of *in silico* mapping.

Epistatic networks

A total of 85 significant epistatic interactions were found when taking into account the three organs and all the traits. Thirteen epistatic interactions were found in leaves, 52 in flower buds and 13 in seeds. Some of these interactions are common to the three organs under study. Sixty-eight interactions were detected in aliphatic GSLs, 13 in indolic GSLs and 4 in total GSLs. An average of 3.5 significant epistatic interactions was found per trait (Fig. S1).

Forty-two interactions were detected between QTLs, being two of them negative. Twenty interactions were detected between QTL9.2 (proposed as GSL-ALK in this work) and other QTLs in traits related to aliphatic GSLs (Fig. 3). The relationship between QTL9.2 and QTL 3.1 (proposed as GSL-OH) was found for the aliphatic GNA, PRO, GER and GIB in the three organs under study. The relationship between QTL9.2 and QTL5.1 (proposed as GSL-PRO) was found for the aliphatic GER, SIN and GNA in the three organs (Fig. 3). In the network controlled by GSL-ALK, interactions between aliphatic and indolic QTLs were observed. For example, QTLs 3.3, 4.1 and 9.1 control the GBS content and the three of them interact with QTL 9.2 in order to produce aliphatic GSLs (Fig. 3).

Discussion

Phenotypic variation in GSL content

Parents of the mapping population had different GSL profiles and concentration. Particularly, parent P_2 has a higher concentration of GRA and a lower concentration of GNA than parent P_1 in the three organs. GRA is found in several *B. oleracea* crops like cauliflower, cabbage and kale, although high levels of GRA equivalent to those found in P_2 ('Early Big' broccoli) are always found in broccoli [35–38]. The effect of sulforaphane, the isothiocyanate derived from GRA, against cancer has been reviewed in detail [8,39]. As a result of these epidemiological and biomedical studies, GRA is now viewed as a quality trait in *B. oleracea* crops to be targeted in breeding programs.

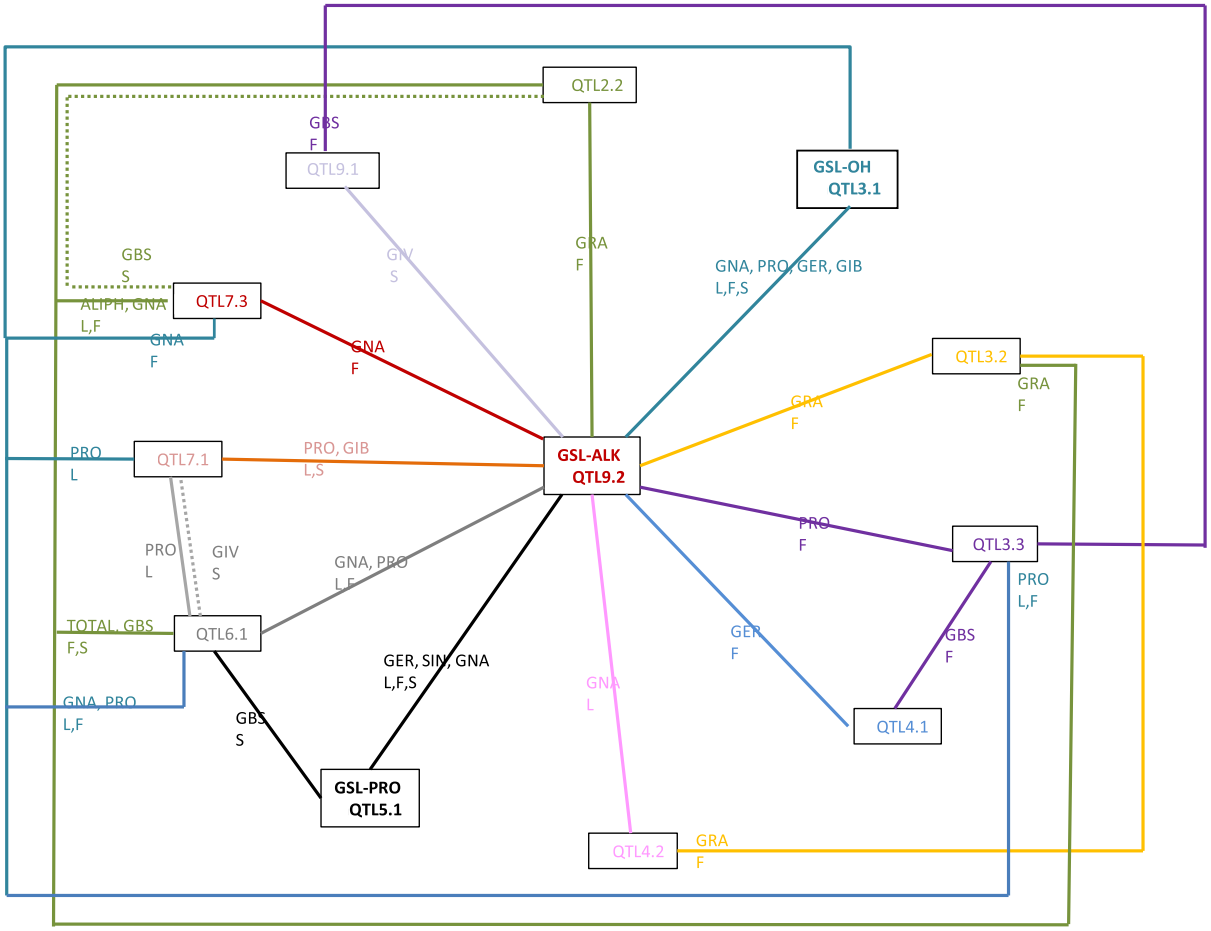

Figure 3. An epistatic network including all the significant relationships of QTL9.2 (GSL-ALK) with other QTLs. Aliphatic glucosinolates: GIV, Glucoiberverin; GIB, Glucoiberin; SIN, Sinigrin; GER, Glucoerucin; GRA, Glucoraphanin; GNA, Gluconapin; PRO, Progoitrin; ALY, Glucoalyssin; GBN, Glucobrassicanapin; ALIPH: sum of aliphatic GSLs; Indolic glucosinolate: GBS, Glucobrassicin; TOTAL: sum of total GSLs. Organs: L, Leaves; F: Flower buds; S: seeds. Continuous lines represent positive epistatic interactions while dashed lines represent negative epistatic interactions.
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Distributions of individual and sums of GSLs were in most cases transgressive. These types of segregations have been described before for GSL content in *Brassica* [40,41] and could be due to new combinations of additive alleles or to epistatic interactions among loci for GSLs, which have already been described [42,43].

Total GSL content varied considerably depending on the organ under study. As it was expected, seeds accumulated the highest GSL content followed by flower buds and leaves. After studying the GSL content in different organs of *A. thaliana*, Brown *et al.* [17] found that seeds had the highest concentration followed by inflorescences, siliques, leaves and roots. Velasco *et al.* [20] found that the GSL content in flower buds was higher than kale leaves [20]. These results may reflect the need to indicate *de novo* synthesis of GSLs and/or mobilization [17].

The GSL profile also varied considerably depending on the organ. In fact, seeds were mostly composed of aliphatic GSLs, whereas indolic GSL were predominant in leaves. Flower buds had an intermediate profile. Besides, flower buds and seeds showed more diversification of aliphatic GSLs, since GIV and ALY were only found in seeds and GER was only found in flower buds and seeds. Agreeing with these results, kale leaves are characterized by high amounts of indolic GSLs during the first plant stages, while aliphatic GSLs are predominant in flower buds and in leaves taken at the end of the vegetative stage [20]. A similar pattern was observed in *A. thaliana*, where seeds are distinguished by unique aliphatic constituents and low level of indolic compounds. After germination, the proportion of aliphatic GSLs declined with age, thus resulting in the predominance of indolic GSLs by the time of senescence [17].

QTLs analysis

Seven out of 20 consensus QTLs determined the content exclusively in one of the three organs under study. Our results suggest that the regulation of genes underlying several QTLs is organ-dependent. Feng *et al.* [43] analysed QTLs for GSL content in leaves and seeds of *B. napus* and found 17 QTLs which were exclusively detected in leaves. Kliebenstein [23] found three organ-specific QTLs for aliphatic GSLs in both leaves and seeds of *A. thaliana*. A similar number was found for indolic GSLs.

Aliphatic GSLs

Several major loci determine the profile and content of aliphatic GSLs in *Brassica* [44]. The GSL-ELONG and GSL-PRO loci regulate the side chain length (Fig. 1B). The presence of 4C-GSL is controlled by a dominant allele of GSL-ELONG (GSL-ELONG+), whereas the presence of 3C-GSL is controlled by a dominant allele of GSL-PRO (GSL-PRO+) [45]. GSL-ALK controls side chain desaturation. The presence of GSL-ALK+ in

3C-GSL determines the production of alkenyl GSL. GSL-OHP catalyzes production of 2-hydroxypropyl GSL, but this GSL was not detected in parents or the mapping population. GSL-OH controls PRO production and its action is conditioned by the presence of GSL-ALK+ [45]. After analyzing parents of the mapping populations, it can be concluded that the genotype of P_1 is GSL-ELONG+, GSL-PRO+, GSL-ALK+ and GSL-OH+, while the genotype of P_2 is GSL-ELONG+, GSL-PRO-, GSL-ALK-. Because P_2 is GSL-ALK- and the presence of GSL-ALK+ is needed in order to produce hydroxylated GSL, the genotype for the locus GSL-OH could not be determined. GSL-ELONG cannot be located into the mapping population, because both parents had the same genotype for this locus. Primer pairs amplifying loci GSL-PROb and GSL-OH designed by Gao *et al.* [15] were located in the mapping population in different positions as those reported by the authors, thus probably indicating an unspecific amplification of PCR products.

Consensus QTL-5.1 controls the amount of three 3C-GSLs: GIB, GIV and SIN. Alleles for increasing 3C-GSLs content are given by P_1. Thus, GSL-PRO would be a good candidate gene for this QTL. This major locus was cloned [14] and mapped at the top of C5 in *B. oleracea* [15]. Position of C5 markers in the map of Iñiguez-Luy *et al.* [22] is inverted with regard to C5 in the map of Gao *et al.* [15]. Taking this into account, the position of QTL-5.1 coincides with that of GSL-PRO. This information together supports the validation of the candidate gene. This QTL also controls the content of two indolic GSLs GBS and NeoGBS. Aliphatic and indolic GSLs are synthesized and subsequently modified by two independent parallel pathways [46]. However, there are cross-talks between both pathways. Wentzell *et al.* [46] found that *GSL.INDOLIC.IV.8* and *GSL.INDOLIC.V.20* QTLs, which control the content of several indolic GSLs in *A. thaliana*, map in the same genomic locations as GSL-AOP and GSL-ELONG loci which control aliphatic GSLs [46].

Consensus QTL-9.2 controls the amount of several GSLs. Alleles for increasing alkenyl GSL content (SIN, PRO, GNA) are given by P_1, while alleles for increasing non alkenyl GSL content (GRA, GER, GIB) are given by P_2 (Fig. 1B). Locus GSL-ALK was studied and cloned by Li and Quiros [13] and mapped in C9 [15] in the same position as QTL-9.2. Consensus QTL-3.1 controls the amount of GNA and its hydroxylated form PRO (Fig. 1B). Curiously, alleles for increasing GNA content are given by P_1which is GSL-OH+, while alleles for increasing PRO content are given by P_2. This makes us think that P_2 is also GSL-OH+. The function of this QTL would correspond to gene GSL-OH. Gao *et al.* [15] mapped this gene in C9, close to GSL-ALK. The position of the gene does not correspond to QTL-3.1. After searching in the whole genome sequence of *B. rapa*, Zang *et al.* [47] and Wang *et al.* [48] found GSL genes homologous to those of *A. thaliana*. Three different copies of gene GSL-OH were found in *B. rapa* due to the triplicate nature of its genome [48]. Several copies of the same genes could also exist in *B. oleracea*.

During the first stage of the development of the core structure of aliphatic GSL (Fig. 1), the gene CYP79F1 metabolizes mono- to hexahomomethionine into their corresponding aldoxime in *A. thaliana* [49]. Primers designed in order to amplify this gene in *B. oleracea* [15] were employed in this work. CYP79F1 mapped in C5, in the same position found by Gao *et al.* [15], but no QTL was found in this position, thus indicating that both parents have the same allele for this gene. Consensus QTL-2.1 controls the content of total aliphatic GSLs in leaves and flower buds and the total GSL content in flower buds, but it does not control the content of any individual GSL, thus suggesting that the gene underlying this QTL may have a regulatory role in the aliphatic GSL pathway. Two R2R3-Myb transcription factors (Myb 28 and Myb 29) positively control biosynthesis of aliphatic GSLs in *A. thaliana* [50] and could be candidate genes for this consensus QTL.

Indolic and aromatic GSLs

In the first stage of the development of the core structure (Fig. 1A) of indolic GSLs, two cytochromes P450 (CYP79B2 and CYP79B3) catalyze the conversion of Trp to indole-3-acetaldoxime in *A. thaliana* [51,52]. Overexpression of CYP79B2 results in an increased accumulation of indole GSLs, specifically 3-indolylmethyl (GBS) and 4-methoxy-glucobrassicin (MeOH-GBS) (not detected in this work). In the next step, CYP83B1 catalyzes the transformation of indole-3-acetaldoxime into to *S*-alkyl-thiohydroximate (Fig. 1A) [53,54]. The Myb transcription factor ATR1 from *A. thaliana* regulates the expression of genes CYP79B2, CYP79B3, and CYP83B1. Overexpression of ATR1 leads to lines with higher levels of total indolic GSLs than wild-type plants [55]. CYP81F2 catalyzes the hydroxylation at position 4 of the indole ring of GBS, which results in the formation of OHGBS and MeOH-GBS [56].

After *in silico* mapping of *A. thaliana* GSL genes, CYP79B2 and CYP79B3 were located inside the confidence interval of consensus QTL-1.2 and QTL-7.4. Both of them determine variation for GBS in seeds, agreeing with a possible high expression of candidate genes CYP79B2 and CYP79B3.

SSRs Gi12 and Ol12-D05 map in both sides of ATR1 gene of *A. thaliana* in chromosome 5 [25]. Gi12 mapped in C9 in our work, where no QTL was detected. Ol12-D05 mapped within the consensus QTL-8.1confidence interval. This QTL determines variation for OHGBS, NeoGBS and total indolic GSL content in the three organs analyzed.

The high apparition of QTLs for indolic GSL content agrees with a high expression of ATR1 candidate gene. Besides, aromatic GNT is also controlled by this QTL. Aromatic GSLs are also a substrate of CYP83B1, regulated by ATR1. These results together suggest that ATR1 could be a possible candidate gene for QTL-8.1.

Consensus QTL-2.1 determines variation for NeoGBS and GBS in flower buds and seeds. Candidate gene CYP81F2, metabolizing the step from GBS to NeoGBS from *A. thaliana*, was found in the confidence interval of this QTL.

The *B. oleracea* whole genome sequencing is currently carried out by using TO1000DH3 as the reference genome. Sequences are being aligned by using mapping population BolTBDH. *B. oleracea* sequencing project will be a great opportunity to link sequences with the QTLs described in this work.

Epistatic networks

Significant epistatic interactions were found for the three organs under study. On the contrary of what was found by *Feng et al.* [43] in *B. napus*, part of the interactions were common among organs. The number of interactions was higher in flower buds, thus indicating a more complex regulation of GSL biosynthesis in this organ. Epistatic interactions for indolic GSLs were less complex than for aliphatic GSLs. 49% of the epistatic interactions detected were between QTLs, thus indicating that variability for GSLs content is determined directly by QTLs and indirectly by interacting with other loci.

Epistatic interactions among GSL-ALK, GSL-PRO and GSL-OH, determine variability for aliphatic GSL content and have been described before (reviewed by Kliebenstein [44]) in *A. thaliana*. They are mediated by transcriptional factors. In this work we have found that GSL-ALK plays a central role in the network of epistatic interactions for aliphatic GSLs, suggesting a possible

regulatory effect of this locus. Indirectly, GSL-ALK also controls the variability for the indolic GSL named GBS, thus indicating cross-talk between indolic and aliphatic pathways. This information supports the results found by Wentzell *et al.* [46] in *A. thaliana*. These authors transformed a null accession for AOP2 and AOP3 genes (GSL-ALK locus) with AOP2 gene from *B. oleracea*, thus resulting in the production of alkenyl GSLs, doubling of total aliphatic GSL content and the induction of aliphatic GSL biosynthetic genes and regulatory genes.

Conclusions

An extensive analysis of QTLs controlling GSL variation in three different organs of *B. oleracea* has been presented. Possible candidate genes for different QTLs have been proposed based on the phenotypic study of the progeny and on the synteny with *A. thaliana*. Epistatic interactions among QTLs have been detected showing a central role of GSL-ALK in determining aliphatic GSL variation and suggesting a regulatory effect of this locus. Further work is going to be carried out in order to validate them and to find new candidate genes for remaining QTLs.

Supporting Information

Figure S1 Complex epistatic interactions in seeds, flower buds and leaves of *Brassica oleracea*. Epistasis network for all analysed glucosinolates. Red lines indicate epistatic interactions for indolic glucosinolates and black lines for aliphatic glucosinolates (a). Epistasis network for individual glucosinolates. In both panels, dot and solid lines indicate negative and positive epistasis, respectively (b).
(PDF)

Table S1 List of metabolic quantitative trait loci (QTL) for glucosinolates in the three plant organs.
(DOCX)

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Author Contributions

Conceived and designed the experiments: PV MEC. Performed the experiments: TS. Analyzed the data: TS PS VMR. Contributed reagents/materials/analysis tools: TS PV PS. Wrote the paper: TS.

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