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Research area: Breakthrough Technologies
phenoVein - A tool for leaf vein segmentation and analysis

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One sentence summary

phenoVein is a user-friendly software tool designed for automated leaf vein segmentation and analysis of leaf vein traits including a model-based vein width determination.
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Abstract

Precise measurements of leaf vein traits are an important aspect of plant phenotyping for ecological and genetic research. Here we present a powerful and user-friendly image analysis tool, named phenoVein. It is dedicated to automated segmenting and analyzing leaf veins of images acquired with different imaging modalities (microscope, macro photography, etc.) including options for comfortable manual correction. Advanced image filtering emphasizes veins from the background and compensates for local brightness inhomogeneities. The most important traits being calculated are total vein length, vein density, piecewise vein lengths and widths, areole area and skeleton graph statistics like the number of branching or ending points. For the determination of vein widths, a model based vein edge estimation approach has been implemented. Validation was performed for the measurement of vein length, vein width and vein density of Arabidopsis thaliana approving the reliability of phenoVein. We demonstrate the power of phenoVein on a set of previously described vein structure mutants of Arabidopsis thaliana (hve-2, ond3, as2-101) compared to the wild type accessions Col-0 and Ler-0. phenoVein is freely available as open source software.

Additional keywords

Arabidopsis, leaf vein density (LVD), MeVisLab, phenotyping, software, vein length per unit area (VLA), vein density (VD), vein width
Introduction

Leaf veins are an important aspect of leaf structure and are responsible for both the mechanical support of leaves and the long-distance transport of water, nutrients and photoassimilates (Onoda et al., 2011; Malinowski, 2013). The molecular mechanisms by which vascular tissues acquire their identities are yet largely unknown (Roschitztardtz et al., 2014) and there is high interest in analyzing and evaluating traits of veins or leaf venation networks and their genetic regulation. The impact of vein density on photosynthesis is a major investigated topic (Sack and Scoffoni, 2013). During the last decade, a positive correlation between leaf venation and photosynthesis has been observed (Brodribb et al., 2007; Sack and Holbrook, 2006). An optimization of photosynthetic rates was demonstrated to occur via a spatial coordination between leaf vein and stomatal densities (Fiorin et al., 2015; Carins Murphy et al., 2014; Zhang et al., 2012). Additionally, there is interest in the impact of vein density on interveinal distances (Dengler et al., 1994; McKown and Dengler, 2009), the effect of climate, habitat, or growth form on vein density (Sack and Scoffoni, 2013; Scoffoni et al., 2015), or vein width with respect to leaf hydraulic conductance (Feild and Brodribb, 2013; Xiong et al., 2015). Other researchers are particularly interested in the evolution from C3 to C4 plants, which requires higher vein density (Gowik and Westhoff, 2011), and led them selecting for variation of vein density within species (e.g. in a mutant collection by Feldman et al., 2014).

Leaf venation studies analyzing traits of veins and venation networks are generally performed on microscopic images of leaves which are properly cleared after harvest. For very small leaves, e.g. the cotyledons or the first leaves (leaf 2 to 5) of Arabidopsis thaliana, basic traits such as total vein length or vein density (vein length per leaf area) can be achieved manually. However, for larger leaves manual vein segmentation may become tedious and at least partially automated analysis is needed for studies on large series of leaf collections. Furthermore, the quantification of vein widths and, in particular, mean values of vein width of certain vein pieces of interest can hardly be achieved manually. Dedicated image processing tools are therefore needed to support researchers for fast and reliable data analysis.

A number of software tools have been published which are either specifically made or adapted to analyze leaf veins. These programs have some common properties, like image processing functionalities for vein/areole segmentation and trait extraction. However, they differ in handling strategies or vein parameter analysis methods. A general overview on plant image analysis tools is
collected in an online database at http://www.plant-image-analysis.org (Lobet et al., 2013). Programs allowing automated or semi-automated analysis of leaf venation parameters are for example a method to extract leaf venation patterns (Rolland-Lagan et al., 2009), the leaf extraction and analysis framework graphical user interface 'LeafGUI' (Price et al., 2011), the leaf image analysis interface 'LIMANI' (Dhondt et al., 2012), the user-interactive vessel generation analysis tool 'VESGEN' (Vickerman et al., 2009; Parsons-Wingerter et al., 2014), and the software network extraction from images 'NEFI' (Dirnberger et al., 2015). Nevertheless, for the analysis of large scale leaf vein phenotyping experiments there are certain needs which are only partly covered by each of the approaches and programs mentioned above. Specifically, the following properties are needed:

a) Automated vein segmentation with optional manual correction.

b) Invariance of the segmentation procedure to inhomogeneous illumination or brightness variations in the leaf image.

c) Automated determination of total vein length and projected leaf area.

d) A well-defined and automated determination of vein widths which is, as far as possible, independent of user chosen thresholds.

e) Ability to process large high resolution images of whole leafs.

f) Full transparency of the source code as well as offline availability of the tool.

In order to provide these functionalities, we developed the user-friendly analysis tool phenoVein. It features automated leaf vein segmentation based on advanced image filtering techniques and includes determination of various vein traits, in particular a model based vein width estimation. phenoVein allows easy and fast visual control and manual correction on the automatically achieved skeleton of the veins enabled by a real time overlay of the segmented leaf vein structures on the original image. The length measurement algorithm of phenoVein was validated against complete manual segmentation. We evaluated the impact of image resolution on the results which has recently been discussed (Price et al., 2014; Sack et al., 2014) and tested whether the orientation (angle) of a leaf on an image may affect the results as suspected from image analysis theory on binary skeleton length measurements (Russ, 2011).

In order to show the powerful phenotyping capabilities of phenoVein, we analyzed the venation traits of leaves of Arabidopsis thaliana at different developmental stages (cotyledons, pooled leaves 1+2, and leaves 6) harvested from previously described venation mutants and corresponding wild type lines: as2-101, ond3, hve-2 vs Col-0 and Ler-0 (Semiarti et al., 2001; Alonso-Peral et al., 2006; Robles et al.,
2010; Pérez-Pérez et al., 2011). We offer the source code of phenoVein to the public as open-source software which can be further adapted or improved (see Materials and Methods for details).

Results and Discussion

Here, we present the design of the image processing pipeline emphasizing the novel features provided by phenoVein. This includes an accuracy analysis of measurements performed with phenoVein and a validation of phenoVein's length and width measurement algorithms. Eventually, different vein traits of a set of leaf vein pattern mutants of *Arabidopsis thaliana* were analyzed using phenoVein. In Table 1 the measured vein traits as well as the terminology and abbreviations used in this work are summarized together with equivalent notations used by other authors.

Leaf preparation and image acquisition

The software phenoVein can deal with all kinds of images of either whole leaves or leaf parts provided that the images show a reasonable contrast between the leaves and the background as well as between the veins and the surrounding areoles. This can be achieved by different approaches. Here, we applied a dedicated method to make *Arabidopsis thaliana* leaves translucent by a clearing protocol. The imaging setup provided light from the bottom of the sample allowing to take pictures with a standard digital single-lens reflex camera (DSLR) placed above the sample (see Materials and Methods). For a precise segmentation of a leaf from the background, any overlap with other leaves or interfering structures (e.g. air bubbles) has to be avoided. In general, it does not matter whether the leaf is brighter or darker than the background, which implies that input images may originate from bright field or dark field approaches. phenoVein is capable to read and process a number of standard image formats such as jpg, png, 8bit-tif, 16bit-tif or bmp.

Image processing sequence

The software phenoVein implements a dedicated image processing sequence consisting of different steps, most of which are depicted in Fig. 1 and Fig. 2:

1. Data preprocessing and image segmentation of leaf from background
2. Filtering of vein structures (vesselness filtering)
3. Binarization and skeletonization
4. Visual inspection and optional manual corrections
5. Vein length determination and evaluation
Vein width estimation

These steps are described and discussed in the following paragraphs. Eventually, all results are written to output files, see Materials and Methods for details.

(1) Data preprocessing and image segmentation of leaf from background. After loading an image (Fig. 1A), a set of preprocessing steps needs to be performed. Firstly, image length scale has to be set in order to allow length and projected area measurements in defined units (e.g. mm or mm², respectively). phenoVein offers three options to determine the image length scale: (i) readout of image meta data (if existing), (ii) manual input by the user or (iii) computation of the image length scale from a known object within the image (e.g. ruler). Secondly, phenoVein uses a gray value image for internal calculations thus input color images need to be converted to gray scale. Depending on the properties of the input images, different color conversion schemes might show different vein-to-areole contrast. To allow optimal contrast selection, the user can choose out of a set of color transformations a desired channel, either selecting one of the RGB (red, green, blue) channels of the input image or via conversion of the input image into one of the single channels of YUV (luminance and color decoding channels) or HSV (hue, saturation, value) color spaces (Russ, 2011). The software also offers the possibility to crop an input image to a user defined region of interest (ROI; Fig. 1A). This allows selecting a whole leaf or part of it for analysis. Images containing multiple leaves can be handled by analyzing each single leaf separately. If the ROI contains background the leaf or the respective subsection is then separated from the background (Fig. 1B,C) by a standard region growing procedure in order to measure the analyzed leaf area, $A$, and to run the vein segmentation only on that part of the image.

(2) Vesselsness filtering. In a next step, the preprocessed input image is filtered with a multi-scale vesselsness filter (Fig. 1D) using the MeVisLab module 'Vesselsness' (MeVis Medical Solutions AG, 28359 Bremen, Germany). This filter enhances pixels which are part of a line or tubular like structure more likely than others by calculating certain measures of the eigenvalues of the Hessian matrix for several scales of the image (Sato et al., 1997; Frangi et al., 1998).

A very handy and desired side effect of the vesselsness filter is robustness against brightness variations in the input image which might for example result from inhomogeneities in illumination or leaf thickness. This becomes obvious when comparing the bright area around the midrib in Fig. 1C with the homogenous areole intensity in Fig. 1D. The vesselsness filter typically shows a decreased contrast at line crossings which is compensated by a morphological closing operation on the filtered image. This
closing operator fills small dark gaps at the vein crossings which usually leads to a reconnection of inadvertently disconnected veins. The filling gap size of the closing operation can be controlled by the user via direct visual inspection.

(3) Binarization and skeletonization. In the subsequent step, the user sets a threshold on the vesselness value at which pixels belonging to veins can be distinguished from those belonging to areoles; this is performed under visual inspection and results in a binary vein mask (Fig. 1E). The obtained mask does not necessarily represent the exact widths of the veins because this depends on the chosen parameters of the vesselness filtering. However, the center of a single mask structure generally represents precisely the center of the underlying vein structure. This allows to apply a skeletonization algorithm which is thinning the vein mask to a one pixel wide skeleton structure (Selle et al., 2002). The skeleton obtained this way preserves vein topology and lengths of the single vein pieces of the binary vein mask (cyan structures in Fig. 1F). At this point, an automatic clean-up process removes small and unconnected vein pieces within the skeleton image (yellow skeleton pieces in Fig. 1F); the minimum size of a single object to be removed can be defined by the user.

(4) Visual inspection and optional manual corrections. Depending on the image quality and possible artifacts such as leaf damage, overlaying trichomes or dirt, the obtained skeleton already gives a good representation of the underlying vein structure. If needed, the user can manually edit the skeleton by removing incorrectly assigned vein pieces or adding missing parts (Fig. 2). phenoVein offers two possibilities to remove undesired veins: (i) veins of a selected region of the leaf can be erased by drawing a closed line using the computer mouse (Fig. 2C) or (ii) by clicking a check mark on single vein pieces which results in recognition and erasing of the vein until a branching point or an ending point is reached (not shown). All removal steps can be cancelled or corrected in case the user accidentally deleted certain structures. The insertion of missing parts is possible with various drawing tools: a linear polyline (as shown in Fig. 2D), an automated live wire (Fig. 3), a spline based line or a freehand line (the two latter options not shown). The live wire is particularly helpful when the user is interested in certain vein structures only (e.g. first or second order veins). In this case, phenoVein offers a feature to mask out all previously detected veins but still allows using them for the live wire segmentation. With this feature, arbitrary vein segments can be selected and redrawn by simply determining the start and end points of the structure of interest and the live wire will deliver the shortest connecting vessel (Fig. 3B). After finalization of the visual inspection, phenoVein provides a calculation of areole size distribution and a visualization by false colors (Fig. 4).
Vein length determination and evaluation. The length of the final skeleton is determined using a weighted pixel counting approach (Russ, 2011). In this method, all horizontal and vertical connections between skeleton pixels are counted and multiplied by 1, whereas all diagonal connections between skeleton pixels are counted and multiplied by \(\sqrt{2}\). This simple method systematically overestimates skeleton length. A correction by a weighing factor of 0.948 considerably reduces the intrinsic bias of the length estimation (Dorst and Smeulders, 1987).

Vein length determination performed with phenoVein was validated by comparison with a manual drawing tool originally designed to measure root length as part of the program package GROWSCREEN_ROOT (Nagel et al., 2009). Both methods were used to segment the veins of several different leaves (Col-0, 10 samples of cotyledon leaves, 5 samples of leaf 1+2 and 3 samples of leaf 6). A high correlation was observed between the results of phenoVein and GROWSCREEN_ROOT (\(R^2=0.9996\), Fig. 5). The manual segmentation took about 20 minutes of pure drawing time per leaf for the leaf 6 samples while, with phenoVein, the results were obtained within 1-2 minutes on a standard PC (Windows 7, Intel i7-2600, 32 GB RAM).

Length measurement of digitized skeletons might possibly depend on the orientation and shape of the measured structures as is known from image analysis theory (Russ, 2011). To investigate the potential impact of orientation on vein length determination, we tested the rotation invariance of phenoVein's length and leaf area measurements. Angle dependence was less than 0.6 %, 0.1 % and 0.8 % for leaf area, skeleton length and vein density, respectively (Fig. 6).

Recently, a discussion emerged in literature dealing with a potential impact of image magnification on vein length and density estimation (Price et al., 2014; Sack et al., 2014). In their study Price et al. claimed that estimates of leaf vein density increase with increasing image magnification which was disproven by Sack et al. Here, we analyzed the effect of image resolution on vein density measured with phenoVein. For this purpose a representative image of an Arabidopsis leaf with high resolution was down sampled to several lower resolutions in order to simulate different magnifications (Fig. 7A). Respective skeleton lengths, leaf areas and the mean width of all veins were determined automatically by phenoVein without any manual corrections. Below a minimal resolution (~<400 dpi) a loss of thin structures results in a considerable decrease of skeleton length (Fig. 7B) while leaf area is not affected (Fig. 7C). In consequence, also leaf vein density requires a minimal image resolution (Fig. 7D). For image resolutions higher than the minimally required resolution leaf vein density remains constant.
which is consistent with the conclusions of Sack et al. that vein density does not increase intrinsically with magnification.

(6) Vein width estimation. After the vein segmentation is completed the average width of either selected vein pieces or of all vein pieces can be measured automatically. A vein piece is the section between two branching points, between a branching point and an ending point, or between two arbitrary end points marked by the user as shown in Fig. 3B. The vein width is defined here as the mean distance between two opposing edges in image intensity separating a vein from the neighboring areole(s). The method to estimate the vein width is a model based vein edge detection approach which fits an enveloping function around the respective vein piece. The mathematical details of this method are given in Materials and Methods.

In most software tools dedicated to analyzing vein traits, the estimation of vein width is not explicitly implemented (e.g. (Rolland-Lagan et al., 2009; Dhondt et al., 2012)). In the software LeafGUI vein width calculation is based on the determination of the vein area, and depends on user defined thresholds (Price et al., 2011; Price et al., 2012). With the software tool NEFI (Dirnberger et al., 2015), the width of veins can be visualized by using a watershed algorithm but no data or validation measurements of the algorithm were presented.

Differently from skeleton length and vein density (Fig. 7, B and D), the estimation of vein widths of Arabidopsis thaliana becomes independent of resolution only above a minimal image resolution of 3000 dpi (Fig. 7E). For a general validation of the model based vein width estimation, artificial images with straight or curved lines were created using CorelDraw (Corel Corporation, version 16.0.0.707) with varying diameters and orientation angles which then were analyzed with phenoVein. The expected structure widths were satisfyingly represented by phenoVein for structures with a minimum width of 5 pixels (data not shown) as can be depicted roughly in the series of pictures of Fig. 7A showing the same image with different resolutions. When veins are represented by too few pixels (e.g. <5 pixels, depending on the smoothness and noise of the image) this may lead to an intrinsic overestimation and thus erroneous value of the vein width as shown in Fig. 7E. This would also impede the estimation of the total areole area, $A_{AR}$, which is calculated here as the projected leaf area, $A$, minus the area covered by the vein, $A_V$ (see Table 1). We are convinced that the method presented here using a model based estimate of vein widths can be considered a reliable and well-defined approach. This method also allows to be extended for a comprehensive error analysis of the fitted model parameters to calculate confidence intervals of the width of each single vein piece, e.g. by bootstrapping (Press, 2007).
Analysis of venation pattern mutants of *Arabidopsis thaliana*

To evaluate the functionality of phenoVein, we compared the venation pattern of known venation-mutant lines (as2-101, hve-2 and ond-3) and their corresponding wild type lines of *Arabidopsis thaliana* (Col-0 and Ler-0) at three different developmental stages: the cotyledons and the pooled leaves no. 1+2 of 14 days-old plants, and the fully developed leaves no. 6 of 30 days-old plants (Fig. 8). We aimed to analyze mutant lines exhibiting varying vein densities as compared to the wild type.

In the mutant *hemivenata* (hve-2), *HVE* encodes a CAND1 protein involved in the auxin signaling pathway (Alonso-Peral et al., 2006; Robles et al., 2010). Compared to Col-0 (Fig. 8A,F,K) the analysis of hve-2 showed lower vein densities in all analyzed developmental stages (Fig. 8, B,G,L and Fig. 9A). The difference in the venation pattern was highest in leaves 1 and 2 in which hve-2 displayed around 71% of Col-0 vein density which is in agreement with (Alonso-Peral et al., 2006). This reduction in vein density was associated with lower areole density but with similar free ending point density as compared to Col-0 indicating that the hve-2 major veins (secondary, tertiary, and quaternary) but no minor veins were interrupted (Table 2). The mean vein width of cotyledons and leaf 6, not accounted by (Alonso-Peral et al., 2006), was significantly increased in hve-2 compared to Col-0 (Fig. 9B) based on slightly smaller fractions of thin veins (Supplementary Fig. S1).

The second analyzed mutant was *ondulata3* (ond3) which is an EMS mutant in Ler-0 background (Pérez-Pérez et al., 2011). We were able to reproduce the results obtained by Pérez-Pérez et al. (2011) by showing that ond3 (Fig. 8D,I,N; Table 2) had a significantly higher vein density in leaves 1+2 and 6, but not in the cotyledons as compared to Ler-0 (Fig. 8C,H,M).

The increase in vein density in the ond3 mutant was around 1.2-fold in leaves 1+2 and 1.7-fold in leaf 6. This increase in vein density was associated with an increase in the areole density. Moreover, ond3 showed a higher ending point density which was 2.2-fold compared to Ler-0 (Table 2). We conclude that the major and minor veins were disrupted in the ond3 mutant because of its higher areole and ending point densities than observed in Ler-0. Interestingly, the different leaf types showed different results with respect to vein width: while the cotyledons and leaves 1+2 had thicker veins, the mean vein width of leaves 6 where smaller compared to Ler-0 (Fig. 9B) due to a higher fraction of thin veins (Fig. S1), possibly compensating for the largely increased vein density in this mutant (Fig. 9A).
To challenge the capability of phenoVein, we analyzed the venation pattern of *asymmetric leaves* (*as2*) mutants. *AS2* is required for the development of veins and of a symmetric and flat lamina. When the gene is knocked out, the leaves will be curled which makes it difficult to acquire images for full leaves using microscopy (Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002) which was also a challenge here as can be seen in Fig. 8O. We found that *as2-101* had lower vein density at the cotyledon stage whereas, in leaves 1+2, the mutant displayed a higher vein density as compared to Ler-0 and, for leaf 6, no significant difference was observed. Since no difference was observed in the areole density between *as2-101* and Ler-0 in leaves 1+2, we hypothesize that the increase in vein density was due to an increase in the number of freely ending minor veins. Indeed, the *as2-101* ending point density was 1.4 fold that of Ler-0 (Table 2). Unlike what was previously shown by Semiarti *et al.* (2001), we found that leaves 1+2 of *as2-101* have higher vein densities than Ler-0 (Table 2). The increase in vein density is due to an increase in minor veins that do not loop, reflected here by a higher ending point density (Table 2). While vein density in *as2-101* showed no clear direction of response for the three leaf stages when compared with Ler-0 (Fig. 9A) the mean vein widths were clearly increased (Fig. 9B). In Fig. S1 it becomes obvious that for leaves 1+2, and in particular leaves 6, the sharp peaks in vein width distribution became flatter with a shift to larger vein widths.

**Conclusions**

We demonstrate the functionality of phenoVein by analyzing the venation pattern of *hve-2, ond3* and *as2-101* mutant lines. Our analysis tool not only enables us to measure vein density but also can obtain important traits like the areole and branching point density as well as a model based estimation of vein widths which can enhance our understanding to the developmental cues controlling venation patterning.

Other unique features contributing to better handling and accuracy of phenoVein are (i) selection of color channels of various color spaces (RGB, HSV or YUV) for optimal vein/areole contrast; (ii) an easy procedure to determine image scaling (pixel size) and (iii) the application of vesselness filtering to enhance linear structures which in particular makes vein detection less sensitive to color/brightness gradients.

For *Arabidopsis thaliana*, the combination of the presented leaf clearing and imaging protocols enables a straight forward segmentation of leaf to background as well as a decent contrast of the leaf veins to
the surrounding areoles. At the same time, trichomes on the leaf do not show up on the image which simplifies the automated segmentation process with phenoVein enormously.

Although automated classification of vein orders was not implemented phenoVein allows a user friendly manual segmentation of certain veins of interest. For plant species that allow establishing adequate vein order classification rules as well as leaf images of sufficient quality, an automated classification is highly promising and a challenge for future software implementations. The current output images of phenoVein allow a subsequent application to already analyzed images if such an algorithm will be developed in the future. Advanced traits like e.g. the spatial distribution about free ending vein points or certain measures of areole size can be extracted subsequently out of the binary skeleton image which is also part of the output files of phenoVein.

The combined use of a camera for image acquisition and phenoVein for venation analysis can be principally applied for other species as well allowing for example the performance of evolutionary studies. Nevertheless, the capability of phenoVein to measure other species automatically strongly depends on image quality. Here, the use of optimized clearing and imaging protocols can significantly improve the automated analysis of phenoVein and avoid tedious manual corrections. Species with extreme characteristics like for example exceedingly high vein densities or veins with high differences in vein width (very thin veins attached to comparatively thick veins) might require customization of phenoVeins segmentation algorithms which is still subject of ongoing investigations.

In short, we provide a new tool which enables fast image acquisition and venation analysis of complete leaves of all sizes. The total time required for the analysis of the 6th 30 day-old *Arabidopsis thaliana* leaf was about 1-4 minutes depending on the leaf size and image quality, making phenoVein a useful tool for large scale analysis such as performing genome wide association mapping.

**Materials and Methods**

**Plant material, growth conditions and leaf clearing**

The following Arabidopsis thaliana lines were analyzed in this study: Col-0 (NASC ID: N22681), Ler-0 (NASC ID: N28445), as2-101(NASC ID: N16274), hve-2 (Robles et al., 2010) and ond3 (Pérez-Pérez et al., 2011). Seeds were sterilized by adding 95% (v/v) ethanol followed by 4% (v/v) HCl and then washed three times with water. Seeds were put on humid freshly prepared soil and vernalized at
4°C for 7 days in the dark. Plants were grown under long-day conditions (16 h of light/8 h of dark) at 23°C with 40% humidity.

The cotyledons and leaves 1+2 (pooled) were harvested from 14-day old plants, while the fully developed leaves 6 were taken from 30-day old plants. The petioles were cut and the leaves were stored in a cold fixation solution composed of methanol-acetic acid (55:2 v/v) for 1-2 day. The leaves were cleared with a mixture of ethanol (water free, 99%)/acetone/methylketone (~94:5:1; v/v/v) (Rotisol; Carl Roth GmbH, Karlsruhe, Germany) by incubation for 4 h at 60°C. After cooling down, the leaves were rinsed two times with water, put upside down onto a microscope slide with water on it and covered with a cover glass.

**Image acquisition**

Images of the cleared leaves were taken with a NIKON D600 single-lens reflex camera (Nikon GmbH, Düsseldorf, Germany) equipped with a macro objective (AF-S VR Micro-Nikkor 105mm f/2.8G IF-ED, Nikon). Samples were positioned close to the minimal focus distance of the objective. With a native sensor resolution of 6016x4016 pixels the pixel size of the images was about 5.7µm at a given field of view of 3.4x2.3cm². The image acquisition was performed in a dark room using transmitted background illumination only. A standard LCD desktop monitor (L568AS, 17", EIZO Europe GmbH, Mönchengladbach, Germany) was diverted as light source allowing us to quickly define custom light field sizes and color compositions. Eventually, a white rectangle with the size of the field of view of the camera was displayed on the screen directly below the sample for image acquisition to avoid ambient scatter light disturbances. The images were saved as Nikon’s raw format (NEF) and converted to 16 bit TIFF images using the software ViewNX-2 (version 2.9.0, Nikon).

**Mathematical description of vein width calculation**

The procedure of the model based vein width estimation is depicted in Fig. 10. In general, we assume that the vein has a certain constant brightness $A$ and the respective areole is darker with a constant brightness $A_0$. The vein edge is defined by the signal transition from the vein to the areole, starting at distance $x_s$ from the vein center (i.e. the skeleton). This transition is assumed to be continuous and is modelled as the right half of a Gaussian function with width $\sigma$. Here, $x$ is the shortest distance to the vein center (next closest skeleton pixel):
The general shape of the vein brightness modeling function $v_e$ is depicted in Fig. 10A. The surrounding
of a vein is composed of all pixels closest to this vein within a maximum distance set by the user
(Fig. 10B, cyan area). The intensities of all pixels within this area are plotted against their individual
shortest distances to the skeleton (Fig. 10C, gray scatter plot). Pixel values with identical shortest
distances to the skeleton $x_k$ are averaged to a single mean value for performance reasons (Fig. 10B,
black scatter plot). The model function $v_e$ with its four parameters $A$, $A_0$, $\sigma$ and $x_s$ is then least squares
fitted to these pixel values (Fig. 10C, cyan curve) using a non-linear Levenberg-Marquardt
optimization routine (Levenberg, 1944; Marquardt, 1963).

The minimized cost function $r(x_k)$ (Eqn. 2) is defined as the weighted difference of the averaged pixel
values $p_k$ and the model function $v_e$ evaluated at the discrete distances $x_k$ (with $k = 1 \ldots$ number of
averaged pixels). This difference is weighted by multiplying with the number of pixels $n_k$ that were
used for averaging and the reciprocal of the distance $x_k$ in order to make the fit more robust (i.e. pixel
values closer to the skeleton have a higher weight than remote pixel values, preventing the optimizer to
run into remote local minima).

$$r(x_k) = \left( \frac{n_k}{x_k} (v_e(x_k) - p_k) \right)^2$$  \hspace{1cm} (Eqn. 2)

If this fit is representing the data well enough (i.e. the sum of squared differences is smaller than a
certain threshold or by visual inspection of the fitting plot), the fitted parameters $x_s$ and $\sigma$ are used to
estimate the mean width $w_i$ for the respective $i$-th vein piece:

$$w_i = 2 \left( x_s + \sqrt{2\ln(2)}\sigma \right)$$  \hspace{1cm} (Eqn. 3)

with $\sqrt{2\ln(2)}\sigma$ being the full width at half maximum factor (FWHM) of the Gaussian function.

Instead of the Gaussian function as an approximation for the vein edge, several other edge functions
(e.g. arccotangens, error function, Heaviside step function) could be used but have not been
implemented in phenoVein. Our choice is supported by the ability of the Gaussian to represent the
involved smoothing processes: the estimation process includes averaging since in most cases the width
will not be strictly constant along a selected vein piece and the limited resolution of camera images leads to partial volume effects causing a blurring of leaf vein images.

**Writing results to output files**

All segmentation results and a summary of the vein analysis parameters are written to a user specified output directory. The output files comprise the following images: leaf mask, binary skeleton, false colored areole size distribution image (Fig. 4), the cropped part of the original image and a skeleton overlay on the original image. Additionally, a comma separated value (csv) file is created containing values of all measured traits as well as a chosen set of derived parameters (Table 1). The csv file can be processed with standard spreadsheet applications.

**Software design and hardware/software requirements**

phenoVein has been implemented as a plug-in within the development environment MeVisLab (MeVis Medical Solutions AG, 28359 Bremen, Germany, free of charge download of "MeVisLab SDK unregistered" at www.mevislab.de). For the current version of phenoVein, an installation of MeVisLab is a prerequisite before phenoVein can be installed. In order to edit and run an adapted version of the source code of phenoVein a specific SDK license of MeVisLab is required. The source code of phenoVein is published in a publicly accessible open source code repository. The link to this repository as well as a signed and executable version of phenoVein can be found at www.phenovein.de.

Minimum recommended hardware and software requirements: a monitor with a minimum resolution of 1650x1050 is recommended as well as a PC with 8GB RAM and Microsoft Windows 7 (www.microsoft.com, Microsoft Corporation, Redmond, WA 98052-6399, USA). phenoVein has been developed and tested with MeVisLab version 2.6.1, earlier versions are not supported. In theory, phenoVein should run under LINUX and OS X as well but was not tested.

**Acknowledgements**

We are thankful to Esther Breuer for supporting us in clearing leaves and making photographic images, and to Siegfried Werth for giving advice in photographing. We thank Dr. José Luis Micol (Universidad Miguel Hernández, Elche, Spain) for providing us with the *hve-2* and *ond3* mutant lines. We would like to thank Kerstin Nagel for providing access to the GROWSCREEN_ROOT software.
**Figure Legends**

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**Figure 6:** Rotation invariance of skeleton length, leaf area and vein density measurement. A, A leaf image (Col-0, leaf 6) illustrating the center of rotation. B and C, Total skeleton length and projected leaf area measured by phenoVein vs rotation angle (0° - 45°). D, Vein density resulting from (B) and (C). The insets show the same data as the full diagrams but with respectively zoomed y-axes.

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**Figure 8.** Photographic images of cleared leaves at different developmental stages of five different genotypes of *Arabidopsis thaliana*. A-E, Cotyledons, F-J, leaf 1 or 2, K-O, leaf 6. Leaves of two wild type accessions are shown in A,F,K, Col-0 and C,H,M, Ler-0, and of three mutants in B,G,L, *hve*-2, D,I,N, *ond3* and E,J,O, *as2-101*. For visualization only, brightness and contrast have been adjusted equally. Scale bar: 2.5mm.

**Figure 9.** Major results of analysis of leaves of *Arabidopsis thaliana* wild types and mutants for cotyledons, leaves 1+2 and leaf 6. An asterisk (*) indicates statistically significant differences (p<0.05)
when comparing a mutant line with its corresponding wild type: 

$hve-2$ vs Col-0, $ond3/as2-101$ vs Ler-0

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Figure 10. Vein edge detection model and estimation of vein widths. A, Sketch of the vein edge model (cf. Eqn. 1). The model function assumes a constant brightness $A$ of pixels representing a vein from its center to the vein edge. The vein edge itself as well as the areole region are modelled by a smooth half-Gaussian function of width $\sigma$, starting at position $x_s$. The areole region is approximated by the tail of the Gaussian function quickly converging to $A_0$. B, Magnified subsection of a leaf photograph with venation network where the image has been inverted to brighten veins over background. Single vein skeleton pieces and their respective environments are highlighted with differently colored pixels. Each colored pixel belongs to its respective next closest skeleton pixel. A vein piece of interest is highlighted and shown in cyan. C, The intensities of all pixels within the cyan area are plotted vs. their respective shortest distances to the middle of the vein (gray scatter plot). For performance reasons, gray values with a unique distance to the skeleton are averaged (black scatter plot). These averaged pixel values are then least squares fitted by the vein edge model (cyan fit curve). The fitted parameters $x_s$ and $\sigma$ are the basis for vein width estimation in Eqn. 3. B, Scale bar: 100$\mu$m.

Supplemental Figure S1: Histograms showing relative frequency of vein widths for all mutants ($hve-2$, $ond3$, $as2-101$) and respective wild types (Col-0 and Ler-0). The frequency of vein widths was weighted bei respective vein lengths.
## Tables

<table>
<thead>
<tr>
<th>Measured traits</th>
<th>Symbol</th>
<th>Definition</th>
<th>Unit</th>
<th>Equivalent terminologies by others</th>
</tr>
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<tbody>
<tr>
<td>Total skeleton length</td>
<td>$L$</td>
<td>mm</td>
<td></td>
<td>Total network length(^1), vascular pattern length(^3)</td>
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<tr>
<td>Projected leaf area</td>
<td>$A$</td>
<td>mm(^2)</td>
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<tr>
<td>Number of vein pieces</td>
<td>$N_v$</td>
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<td>No. of edges(^1), number of vascular elements(^3)</td>
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<td>Piecewise vein length(^*)</td>
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<td>edge length(^1)</td>
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<td>Number of branching points</td>
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<td>No. of nodes(^1)</td>
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<tr>
<td>Number of ending points</td>
<td>$N_{EP}$</td>
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<td></td>
<td>Free-ending veins (FEV)(^2)</td>
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<tr>
<td>Number of areoles</td>
<td>$N_{AR}$</td>
<td></td>
<td></td>
<td>No. of areoles(^1), number of loops(^4)</td>
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<tr>
<td>Single interstitial areas(^**)</td>
<td>$A_i (i = 1...N_{AR})$</td>
<td>mm(^2)</td>
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### Derived traits

<table>
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<td>Leaf vein density</td>
<td>$D_V$</td>
<td>$= L / A$</td>
<td>mm mm(^{-2})</td>
<td>Vein length per leaf area (VLA)(^2), vascular density(^3)</td>
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<td>Branching point density</td>
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<td>Ending point density</td>
<td>$D_{EP}$</td>
<td>$= N_{EP} / A$</td>
<td>mm(^{-2})</td>
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<td>Areole number density</td>
<td>$D_{AR}$</td>
<td>$= N_{AR} / A$</td>
<td>mm(^{-2})</td>
<td>Number of vein areoles per leaf area (VAA)(^2)</td>
</tr>
<tr>
<td>Total vein area</td>
<td>$A_V$</td>
<td>$= \sum L_i W_i$</td>
<td>mm(^2)</td>
<td>Total network 2D area(^1)</td>
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<td>Total areole area</td>
<td>$A_{AR}$</td>
<td>$= A - A_V$</td>
<td>mm(^2)</td>
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<tr>
<td>Mean vein width</td>
<td>$W_{mean}$</td>
<td>$= A_V / L$</td>
<td>mm</td>
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**Table 1:** Summary of measured leaf venation traits, derived parameters, terminology and abbreviating symbols used here as well as equivalent terms used by other authors. \(^*\)A piecewise vein length results from the respective piecewise skeleton length between two branching points or a branching point and an ending point. \(^**\)A single interstitial area differs from the respective single areole area since the interstitial area includes most of the vein area as well (except for the area covered by the skeleton pixels). References: (1) Price *et al.*, 2011, (2) Sack and Scoffoni, 2013, (3) Dhondt *et al.*, 2012, (4) Rolland-Lagan *et al.*, 2009.
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<th>Leaf type</th>
<th>Trait</th>
<th>Col-0</th>
<th>hve-2</th>
<th>Ler-0</th>
<th>ond3</th>
<th>as2-101</th>
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<td>Cotyledons</td>
<td>$D_V$</td>
<td>1.60 ± 0.08</td>
<td>1.41 ± 0.13*</td>
<td>1.48 ± 0.12</td>
<td>1.39 ± 0.08</td>
<td>1.36 ± 0.05*</td>
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<td>$D_{BP}$</td>
<td>0.71 ± 0.16</td>
<td>0.56 ± 0.11*</td>
<td>0.72 ± 0.20</td>
<td>0.68 ± 0.12</td>
<td>0.58 ± 0.05</td>
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<td>$D_{EP}$</td>
<td>0.20 ± 0.14</td>
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<td>$D_{AR}$</td>
<td>0.47 ± 0.09</td>
<td>0.35 ± 0.09*</td>
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<td>0.42 ± 0.09</td>
<td>0.39 ± 0.05</td>
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<td>Leaves 1+2</td>
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<td>2.87 ± 0.14</td>
<td>3.56 ± 0.21*</td>
<td>3.25 ± 0.21*</td>
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<td>$D_{BP}$</td>
<td>3.39 ± 0.33</td>
<td>1.60 ± 0.15*</td>
<td>3.58 ± 0.38</td>
<td>6.35 ± 0.74*</td>
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<td>$D_{EP}$</td>
<td>0.87 ± 0.27</td>
<td>0.76 ± 0.13</td>
<td>0.95 ± 0.18</td>
<td>1.84 ± 0.25*</td>
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<td>$D_{AR}$</td>
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<td>1.37 ± 0.17</td>
<td>2.37 ± 0.40*</td>
<td>1.59 ± 0.30</td>
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<tr>
<td>Leaf 6</td>
<td>$D_V$</td>
<td>2.61 ± 0.38</td>
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<td>2.64 ± 0.39</td>
<td>4.37 ± 0.34*</td>
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<td>$D_{BP}$</td>
<td>2.67 ± 0.93</td>
<td>1.84 ± 0.42*</td>
<td>3.08 ± 0.98</td>
<td>8.70 ± 1.45*</td>
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<td>$D_{EP}$</td>
<td>0.94 ± 0.26</td>
<td>0.81 ± 0.18</td>
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<td>1.75 ± 0.13*</td>
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<td>$D_{AR}$</td>
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<td>0.56 ± 0.16*</td>
<td>1.15 ± 0.41</td>
<td>3.53 ± 0.74*</td>
<td>1.03 ± 0.39</td>
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**Table 2:** Summary of venation pattern results for traits $D_V$ (vein density, unit: mm mm$^{-2}$), $D_{BP}$ (branching point density, unit: mm$^{-2}$), $D_{EP}$ (ending point index, unit: mm$^{-2}$) and $D_{AR}$ (areole number density, unit: mm$^{-2}$). Values are mean values ± SD, n = 10. An asterisk (*) indicates statistically significant differences (p<0.05) when comparing a mutant line with its corresponding wild type: hve-2 vs Col-0, ond3/as2-101 vs Ler-0 (standard Student’s -test). (1) Some of the as2-101 leaves 6 were cut in order to allow flat imaging. For this reason, the number of free ending points $N_{EP}$ found by phenoVein was increased inadvertently by the cut veins. These cases were corrected manually by subtracting the undesired ending points.

**Supplemental Table S1:** Merged output of phenoVein for cotyledons, leaves 1+2 and leaf 6 for all mutants (hve-2, ond3, as2-101) and wild types (Col-0, Ler-0).
References


Brodribb TJ, Feild TS, Jordan GJ (2007) Leaf maximum photosynthetic rate and venation are linked by hydraulics. Plant Physiology 144: 1890-1898


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